INNATE IMMUNE RESPONSES IN CNS INFLAMMATION

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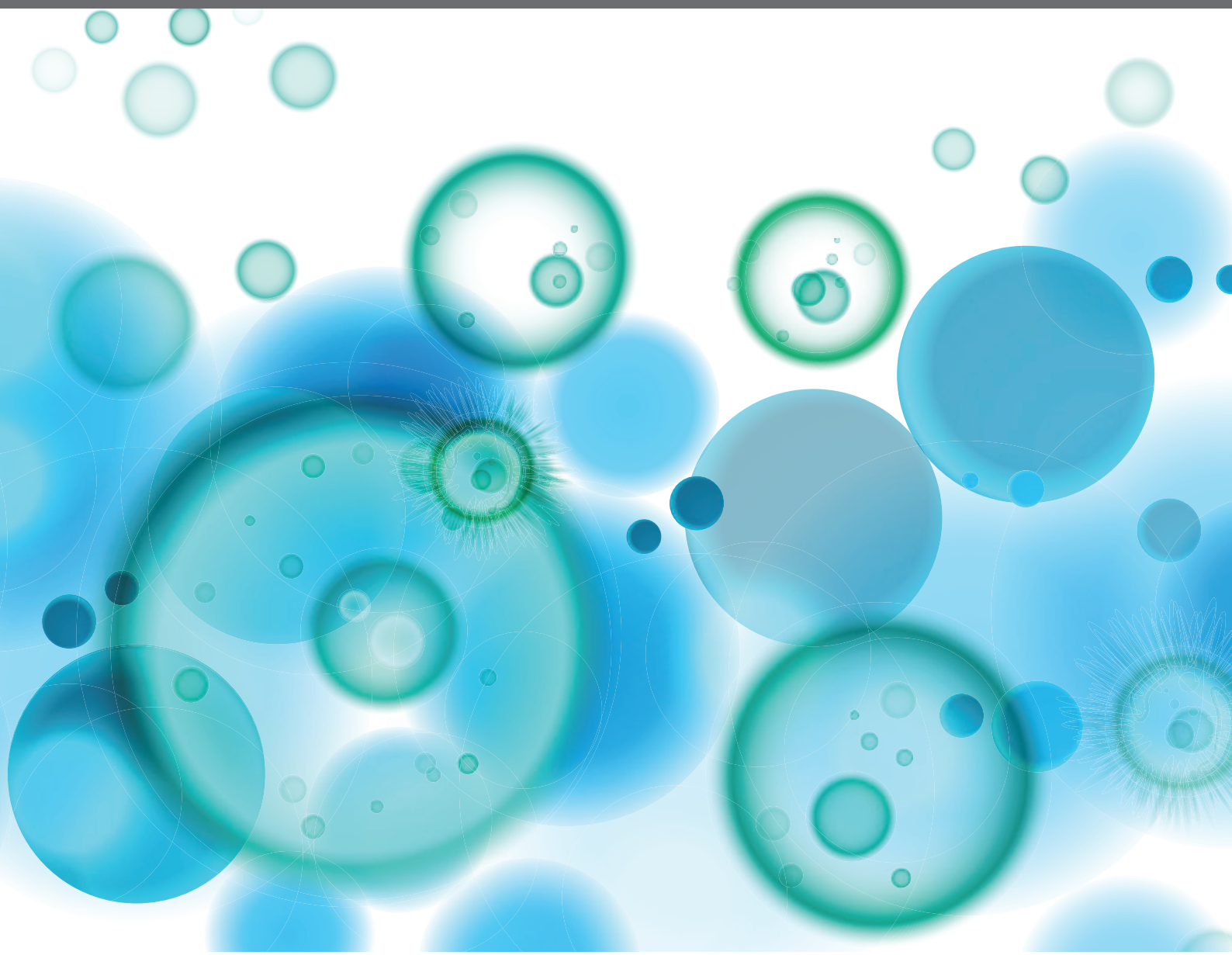

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INNATE IMMUNE RESPONSES IN CNS INFLAMMATION

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REVIEW
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Molecular Mechanisms Modulating the Phenotype of Macrophages and Microglia

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Macrophages and microglia play crucial roles during central nervous system development, homeostasis and acute events such as infection or injury. The diverse functions of tissue macrophages and microglia are mirrored by equally diverse phenotypes. A model of inflammatory/M1 versus a resolution phase/M2 macrophages has been widely used. However, the complexity of macrophage function can only be achieved by the existence of varied, plastic and tridimensional macrophage phenotypes. Understanding how tissue macrophages integrate environmental signals *via* molecular programs to define pathogen/injury inflammatory responses provides an opportunity to better understand the multilayered nature of macrophages, as well as target and modulate cellular programs to control excessive inflammation. This is particularly important in MS and other neuroinflammatory diseases, where chronic inflammatory macrophage and microglial responses may contribute to pathology. Here, we perform a comprehensive review of our current understanding of how molecular pathways modulate tissue macrophage phenotype, covering both classic pathways and the emerging role of microRNAs, receptor-tyrosine kinases and metabolism in macrophage phenotype. In addition, we discuss pathway parallels in microglia, novel markers helpful in the identification of peripheral macrophages versus microglia and markers linked to their phenotype.

Keywords: macrophages, microglia, central nervous system, inflammation, molecular, microRNA, metabolism

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INTRODUCTION

Macrophages in the central nervous system (CNS) play important homeostatic and immune defense roles (1). While microglia originate from early yolk sac myeloid progenitors and become self-regenerating CNS-resident cells (2–4), macrophages originate from peripheral blood monocytes. Microglia are essential for appropriate synaptic pruning during development (1). During steady state condition, microglia also facilitate learning and memory and remove cellular or other debris. Upon CNS infections and injury, microglial activation and peripheral macrophage recruitment and activation occur. Both macrophages and microglia have the capacity to recognize pathogens or injured cells, activating phagocytic, antigen-presenting and cytokine/chemokine secretion functions that modulate immunity and mediate pathogen or cellular debris elimination (1). Macrophages and microglia also contribute to resolution stages of inflammation and tissue regeneration *via*

switching to anti-inflammatory cytokine patterns, promoting intercellular matrix synthesis and angiogenesis. The complexity of macrophage function is mirrored by the existence of varied, plastic and multilayered macrophage phenotypes *in vivo* (5). However, for simplicity, a model of inflammatory/classical M1 and resolution/alternatively activated M2 macrophages has been widely used.

Understanding the molecular programs that define inflammatory versus resolution phenotypes provides the opportunity to target and modulate these cellular programs to control the excessive inflammation typical of chronic inflammatory CNS conditions such as Multiple Sclerosis (MS) and CNS injury. In recent years, our understanding of how environmental signals are integrated into macrophage phenotype has greatly advanced. The classic roles of NOTCH, PI3K/AKT, MYC, PPAR, and interferon regulatory factors (IRFs) in macrophage polarization have been further established while prominent roles for metabolism, microRNAs (miRNAs) and receptor-tyrosine kinases (RTKs) are now clear. The ability to distinguish microglia from CNS macrophages and inflammatory vs. resolution macrophages has greatly advanced with the discovery of new markers. In this review, we discuss these findings and present the current understanding in the field of molecular mechanisms and markers of inflammatory versus resolution macrophages, as well as therapeutic implications of macrophage modulation for the CNS autoimmune disease MS.

MACROPHAGES AND MICROGLIA: SIMILAR BUT NOT THE SAME (DEVELOPMENTAL ORIGIN, FUNCTIONS, AND MARKERS)

Microglia and macrophages have many functions in common. They both help to maintain homeostasis during embryogenesis and into adulthood (6–8). Additionally, both cells are sentinels in their respective environments, scanning for foreign invaders and pathogens (9–12). Both cell types also differentiate into a spectrum of proinflammatory to proregenerative subsets in response to injury or insult (13, 14). In addition to the roles microglia play in fighting infection and clearing debris *via* phagocytosis, microglia are also important in neuronal proliferation and differentiation and the formation and pruning of synaptic connections in neuronal networks (15, 16). Based on the specific genes expressed in microglia and the subset of functions unique to microglia, one can postulate that other tissue-specific macrophages have roles exclusive to their tissue that monocyte-derived macrophages cannot replace.

Monocyte-derived CNS macrophages and microglia have similar morphologies and phagocytic functions but their origins are distinct. Until a short time ago it was believed that solely circulating monocytes replenish tissue macrophage populations, including those in the CNS, but this view is now rejected based on new reports in the literature (17, 18). Although bone marrow derived monocytes can enter tissues such as the CNS and differentiate into macrophages, microglia and other tissue macrophages are now thought to originate most exclusively from earlier embryonic progenitors (19). Embryonic hematopoiesis consists of three main waves, namely primitive, transient definitive and definitive hematopoiesis. Primitive hematopoiesis originates from yolk sac blood islands around embryonic day (E)7, yielding progenitors as early as E7.5 (19). The transient definitive hematopoiesis wave starts around E8 when hemogenic endothelium develops, producing erythromyeloid precursors (EMPs) (19). Upon establishment of circulation starting at E8.5, EMPs migrate to the fetal liver where they support definitive hematopoiesis (19). EMPs will also eventually migrate and support bone marrow hematopoiesis in the adult.

Three models of fetal microglia and tissue macrophage ontogeny have been proposed (19). Two models favor the view that most microglia but few of other tissue macrophages derive from the early wave of primitive hematopoiesis in the yolk sac (20, 21). The remaining model instead supports the view that EMPs from transient definitive hematopoiesis give rise to most microglia and other tissue macrophages (22). All these models are all in agreement on the embryonic origin of microglia, with little or no contribution from monocytes. Sublethally irradiated C56BL/6 CD45.2$^+$ newborn mice reconstituted with hematopoietic cells isolated from CD45.1$^+$ congenic mice had 95% microglia were of host origin (CD45.2$^+$ cells gated on CD11b$^+$CD45^{int}, then Ly6G$^-$F4/80$^+$) 3 months after transplant, while over 30% of circulating leukocytes were of donor origin (2). These data support the idea that microglia are a distinct population not populated/replenished by circulating monocytes. Kierdorf and colleagues added to our knowledge by identifying the earliest yolk sac progenitors with the potential to become microglia to be CD45$^-$ c-Kit$^+$ erythromyeloid precursors (EMPs), and these differentiated into Iba-1$^+$ Cx3cr1$^-$ cells with microglial-like morphology (3). Two transcription factors important in driving EMPs to differentiate into microglia and CNS macrophages are PU.1 and IRF-8. *Pu.1* gene deficient animals lacked microglia completely, while mice-lacking the *Irf8* gene had significantly reduced numbers of microglia (3). Upon analysis of the yolk sac progenitors, they found that PU.1 is necessary for the initial transition from EMPs (c-KIT$^+$) to early microglial precursors (CD45$^+$ c-KIT^{lo} CX3CR1$^-$); whereas IRF-8 acts downstream of PU.1 and plays a role in the transition from early to mid-stage microgliogenesis (CD45$^+$ c-KIT$^-$ CX3CR1$^+$) (3). Another molecule important in shaping microglial development is negative regulator of reactive oxygen species (NRROS, aka LRRC33). *Nrros* gene deficient mice lack normal CD11b^{hi}CD45^{lo} microglia, and CX3CR1-driven deletion of *Nrros* leads to impaired expression of *Sall1* (lineage-specific transcription factor important for maintenance in adult microglia) and other microglial genes needed for microglial development and function (23). Interestingly, *Nrros*$^{-/-}$ mice have normal numbers of myeloid progenitor cells in the CNS at E10.5, but the CD11b^{hi}CD45^{lo} microglial population was largely absent by E14.5, suggesting NRROS is important in early microglial development.

Can Microglia Be Differentiated from Peripheral Origin Macrophages?

Discriminating between peripheral macrophages and microglia has been a difficult technical issue. Microglia and macrophages

share many markers such as CD11b, F4/80, CX3CR1 and IBA1 (13). High levels of CD45 expression ($CD45^{hi}$) have long been used to discern peripheral macrophages from microglia, which express lower levels of CD45 (24). However, peripheral macrophages may downregulate CD45 once in the CNS or in response to injury (25). CX3CR1 (aka fractalkine receptor) is expressed by microglia throughout development and into adulthood (26). Since it is not expressed by other CNS-origin cells (27), CX3CR1 can be used to detect microglia in naive tissues. During inflammation, however, peripheral macrophages, monocytes and T cells also express CX3CR1 and infiltrate the CNS (28). The use of irradiated *Cx3cr1*–green fluorescent protein (GFP) knock-in mice (27, 28) as recipients of WT bone marrow yields a model in which only microglia express GFP and peripheral macrophages can be detected by use of donor markers. Another model, a tamoxifen-inducible Cre mouse line crossed with a red fluorescent protein (RFP) Cre reporter mouse line ($Cx3cr1^{YFP\text{-}CreER/wt}$:$R26^{RFP}$), can differentially label microglia and recruited macrophages by pulsing mice with tamoxifen and then following the YFP^+RFP^+ labeled cells (29). Macrophages will turnover quickly and lose RFP expression, while microglia will retain RFP expression because they are long-lived. Besides a marker and chemoattraction role, CX3CR1 has an essential role in promoting a resting microglial phenotype and neuroprotection (30). $Cx3cr1^{-/-}$ mice had worsened neurologic dysfunction in the EAE model (31). In contrast, post-spinal cord injury (SCI) recovery was enhanced in $Cx3cr1^{-/-}$ mice (32), suggesting context and CX3CR1 expression on cells beyond microglia and macrophages influence disease outcomes. Soluble CD163, which is cleaved from CD163 on macrophage/microglia membranes, may also be a marker for MS or for inflammation in general (33–35). In addition, many markers co-expressed by peripheral macrophages and monocytes are present on activated microglia as well. For example, CD169 is a marker for macrophages (13) that was recently identified on early activated microglia in MS and EAE lesions (36). MERTK is another common marker for many tissue specific macrophages including microglia (13).

The most prominent difference between microglia and macrophages appears to be their developmental origin. One marker specific to microglia that does not stain infiltrating peripheral immune cells is TMEM119 (25, 37). TMEM119 protein is expressed on all microglia by postnatal day 14 (P14) and remains expressed in post-sciatic nerve crush injury, LPS injection and optic nerve crush injury (25). Specifically, $Ccr2^{RFP/+}$ mice (in which RFP is only expressed in infiltrating monocytes) showed $IBA1^+TMEM119^-$ cells were mostly RFP^+ and RFP^+ cells were never $TMEM119^+$, suggesting TMEM119 is a stable resident microglia marker that does not recognize infiltrating macrophages. Importantly, TMEM119 is a marker for both mouse and human microglia (25) and is maintained in MS lesional tissue (38). The availability of this marker has revealed that many microglial markers are induced, while macrophage markers are suppressed, in peripheral macrophages that infiltrate the CNS (38). FCRLS is another highly expressed gene specific to murine microglia, but there is no ortholog in humans (39). Another microglial-specific marker P2RY12, a purinergic receptor associated with homeostatic microglia not detected on lymphatic tissue (38), is highly expressed in normal white matter of MS patients. However, as microglia become stimulated in active MS lesions, P2RY12 is sometimes downregulated, while proinflammatory genes such as iNOS and CD86 are upregulated (38), confounding the distinction of microglia from peripheral cells during inflammation. Since another report found that P2RY12 remains elevated after EAE induction (39), this may be a human/mouse model difference. TMEM119 seems to be the most discriminatory of the new markers and has allowed to sort microglia based on their developmental origin. These studies have revealed other genes specific of this population that may lead to additional methods for microglial detection (25).

MACROPHAGE/MICROGLIA PHENOTYPES, FUNCTION, AND NOMENCLATURE

The dual role of macrophages and microglia in promoting inflammation vs. resolution is mediated by distinct gene expression programs and macrophage phenotypes. This inflammatory phenotype is induced by ligation of pathogen recognition receptors (PRRs), such as Toll-like receptors (TLRs), on macrophages to pathogen- or danger-associated molecular patterns (PAMPs or DAMPs) from microbes or damaged/dying cells (40). These signals may be combined with inflammatory cytokines produced by Th1 cells, such as IFN-γ. *In vitro*, this phenotype has been modeled by stimulation of bone marrow-derived macrophages or microglia with LPS (+IFN-γ). Macrophages activated in this manner have long been known as classically activated or M1 macrophages (41). In 2014, in an effort to reach consistency and clarity in the field, novel nomenclature that follows the letter M by a parenthesis enclosing the stimuli used for activation was proposed (42). For example, M1 macrophages stimulated with LPS and IFN-γ are indicated as M(LPS + IFN-γ) while macrophages stimulated with LPS alone would be labeled M(LPS). Macrophages differentiated with GM-CSF, or M(GM-CSF) macrophages, have also been described to have a proinflammatory phenotype (43). This nomenclature is providing an extremely useful standardized tool to communicate macrophage experimental data. In this review, we use this nomenclature when the specific stimulation is known, while the simple M1 vs. M2 notation is used when referring to a general inflammatory vs. resolution/alternatively activated phenotype of macrophages.

Functionally, M1 macrophages are responsible for fighting bacterial infections and adopt a phenotype characterized by microbicidal, antigen-presenting and immune potentiating abilities. This is accomplished by induction of inducible nitric oxide synthase (iNOS, encoded by the *Nos2* gene), which synthesizes microbicidal nitric oxide (NO) in most rodent models (44, 45). It is important to note, however, that iNOS induction does not occur in human macrophages. In addition, M1 macrophages recruit additional cells to the site of infection and bridge innate and adaptive immunity. This is accomplished by induction of chemokines and inflammatory cytokines interleukin (IL)-6, IL-12, IL-1β, IL-23, and TNF-α that recruit immune cells to sites of infection and polarize them to type I responses and by CD80 and CD86

costimulatory molecule expression to prime T cells (42, 46). Our lab has recently characterized CD38 as a marker that is increased in inflammatory murine bone marrow-derived M(LPS + IFN-γ) macrophages and decreased in M2 macrophages compared to untreated M0 macrophages (47). CD38 upregulation is also observed in a sepsis model (47) as well as Experimental Autoimmune Encephalomyelitis (EAE), the mouse model of MS (48). Given that CD38 is a surface marker that allows live cell sorting for downstream applications, it provides an advantage over intracellular markers such as iNOS. Although CD38 is known to be an ectoenzyme that catalyzes conversion of NAD to ADP-ribose and induces calcium signaling inside the cell (49), its exact role in inflammatory phenotype is unknown. However, it appears to play an important role, as CD38 induction by LXR and NAD depletion is necessary to limit bacterial uptake and inflammatory cytokine production (50). Future studies will be necessary to determine whether CD38 plays a similar role in human macrophages. For a listing of current inflammatory phenotype markers in macrophage/microglia, see **Table 1**. Like macrophages, microglia secrete inflammatory cytokines IL-1β, IL-6, IL-12, and TNF-α when exposed to LPS (+IFN-γ). Similarly, they upregulate iNOS and CD38 (51–56). Although most studies have been done in murine microglia, LPS + IFN-γ also induces M1 phenotype in primary human microglia (57).

The evolution from acute inflammation to a resolution phase occurs as initial neutrophils undergo apoptosis and monocytes, which will switch to a resolution/M2 phenotype, predominate in the tissue (58). Key lipid mediators in promoting the resolution phase include classical eicosanoids, phospholipids and sphingolipids, endocannabinoids (eCBs) and specialized proresolving mediators (SPMs) (59). The classical eicosanoids thromboxanes (TX) and prostacyclins antagonize inflammation while phospholipids and sphingolipids such as phosphatidylserine (PtdlSer), when recognized by macrophages, promote M2 switch (60). eCBs such as N-arachidonoylethanolamine (AEA) and N-palmitoylethanolamine (PEA) have immunomodulatory roles, particularly in neuroinflammation (61). Last, but not least, SPMs have major proresolution activities. Main SPMs include lipoxins (LX)A4 and LXB4, resolvins (Rv) RvD1-6 derived from docosahexanoic acid (DHA), RvE1-3 derived from eicosapentanoic acid (EPA), protectin D1 and maresins. In particular, maresins have been shown to shift to a resolving macrophage phenotype (62), which can also be induced by exposure to Th2 cytokines like IL-4 and IL-13, parasites, fungal cells, apoptotic cells, immune complexes, adenosine, or transforming growth factor (TGF)-β (63). *In vitro*, four M2 type macrophages were initially described, corresponding to macrophages stimulated with IL-4 or IL-13 (M2a), IL-1R ligands or immune complexes (M2b), IL-10, TGF-β or glucocorticoid (M2c) and IL-6 and adenosine (M2d) (64). This notation has now been replaced with the M(stimulus) nomenclature that clearly defines the inducing stimulus (42). For, example, macrophages stimulated with IL-4 are indicated as M(IL-4) and pro-M2 macrophages differentiated with M-CSF are called M(M-CSF).

TABLE 1 | Inflammatory phenotype macrophage/microglia markers.

		M(LPS + IFNγ)	M(IL-4)
Transcription factors	Mouse	pSTAT1	pSTAT6, Irf4
	Human	pSTAT1, IRF1, IRF5	IRF4
Amino acid metabolism	Mouse	iNOS	Arginase-1
	Human	IDO1	
Scavenger receptors	Mouse		Mrc1 (CD206), CD163
	Human		MRC1 (CD206), CD163
Cytokines	Mouse	TNFα, IL6, IL12A, IL23A, IL27	
	Human	TNF, IL1B, IL6, IL12A, IL12B, IL23A	
Others	Mouse	CD38, CD80, CD86, FPR2	RELMα (FIZZ1), CHI3L3 (YM-1), ALOX15, EGR2, c-MYC
	Human	CD40	ALOX15

It is currently being debated whether resolution/wound healing macrophages are an evolution of initial inflammatory macrophages under the changing local environment or, rather, they originate from newly recruited peripheral monocytes. Similarly, resolution spectrum macrophages may revert to an inflammatory phenotype if new inflammatory stimuli are encountered (65). Resolution macrophages suppress IL-12 secretion and may secrete anti-inflammatory mediators IL-10, TGF-β, IL-1R antagonist (IL-1RA), and decoy IL-1R II (66). In addition, these macrophages express arginase-1 instead of iNOS, switching arginine metabolism from production of NO to ornithine and polyamines for collagen and extracellular matrix synthesis (67). M2 markers arginase-1, resistin-like alpha (RELMα/FIZZ1), and chitinase 3-like protein 3 (CHI3L3/YM1) are detected in murine but not human macrophages (68–70), although it should be noted that a portion of murine M1 stimulated cells also upregulates arginase-1 (42, 47). Murine M(IL-4), but not M(LPS + IFN-γ), bone marrow derived macrophages (BMDMs) can be identified by flow cytometry detection of the intracellular transcription factor EGR2 (47), which is related to the M2 transcription factor C-MYC. C-MYC (47, 71) and CD206 (41) are M2 markers common to murine and human macrophages. CD169 [aka sialic acid binding Ig-like lectin-1 (SIGLEC-1)] in certain *in vivo* macrophage populations (72) and tyrosine-protein kinase Mer (MERTK) in M2c macrophages (73) have also been identified as markers useful in flow cytometry (13, 74). CD169 and MERTK are also expressed in microglia.

Does macrophage and microglial phenotype modulate neuroinflammation and MS? The hypothesis that an inflammatory phenotype in macrophages or microglia is damaging to the CNS while a resolving phenotype contributes to neuroregeneration was introduced by the Popovich group in the late 2000s (75). They found that while inflammatory macrophage responses cause neurotoxicity, resolving macrophage responses instead promote neuron viability and regenerative growth toward repair (75). Evidence supporting this hypothesis, which has important therapeutic implications, has since accumulated in multiple neuroinflammatory paradigms. We will discuss below the latest

evidence for a role of macrophage and microglial phenotype on modulation of CNS neuroinflammation and remyelination in multiple sclerosis and its animal model EAE.

Activated microglia are one of the first observations very early in MS while both monocyte-derived macrophages and activated P2RY12$^{-/lo}$ microglia are found later in active lesions (38). Large numbers of macrophages and microglia, coinciding with myelin breakdown, are a hallmark of the acute MS lesion (76). High oxidative activity and expression of IL-1β and IL-23p19, all characteristic of inflammatory macrophages and microglia, are observed in these lesions. Exacerbated and fast-progressing EAE occurs in mice with a microglial-specific Nr4a1 deficiency which results in increased microglial activation and NO production, consistent with the damaging M1 responses. Neurodegenerative microglia characterized by a TREM2-APOE pathway signature, are generated after neuron phagocytosis, further establishing a neurodegenerative cycle (77). Inflammatory microglia do in turn induce a subtype of inflammatory astrocytes, termed A1, which can no longer sustain neurons and induce neuron and oligodendrocyte cell death. A1 astrocytes are abundant in the CNS of various neurodegenerative diseases, including MS lesions (78).

So, what about resolution? It is unclear what exactly makes an active MS lesion evolve toward resolution. PtdlSer, a phospholipid present in myelin, may play a role. PtdlSer liposomes suppress NO and inflammatory cytokine production in macrophages and *in vivo* treatment ameliorates EAE (79). The early resolving lesion contains instead lipid-laden, aka foamy, macrophages or microglia spread throughout the lesion and forming a layer at the lesion edge. There is no evidence of remyelination in early resolving lesions but examples of remyelination in late resolving lesions are fairly common (80). Interestingly, foamy macrophages or microglia abound within these remyelinated areas (80, 81), as if consistent with a reparative role. Consistent with a beneficial role of resolution macrophages, mice deficient in the M2-promoting factor SOCS3 suffer from chronic and more severe EAE while adoptive transfer or promotion of resolution spectrum macrophages suppresses EAE disease (82–84). Besides being less neurotoxic and chemotactic, M2 macrophages may play an active proregeneration role. Consistent with the latter, a shift from inflammatory to resolving phenotype in microglia and infiltrating macrophages is observed during remyelination (81). The shift to M2 phenotype drives oligodendrocyte differentiation in an activin A-dependent manner (81). Taken together, these findings provide a framework for the importance of inflammatory macrophage and microglial phenotype in driving MS neuroinflammation and the therapeutic promise of promoting opposing M2 responses.

CLASSIC MOLECULAR MECHANISMS MODULATING MACROPHAGE PHENOTYPE

The distinct phenotypic features of inflammatory vs. wound-healing macrophages are controlled by a network of molecular pathways that relay environmental signals *via* signaling cascades to impact gene expression and cellular metabolism (see **Figure 1** for a summary of molecular pathways that modulate macrophage phenotype). PI3K/AKT, NOTCH, PPARs, MYC, and IRFs have been known to modulate macrophage phenotype. Novel data demonstrating crucial roles for metabolism, RTKs, miRNA and epigenetic modifications will be discussed in a subsequent section.

PI3K/AKT

The PI3K/AKT pathway is activated in response to environmental stimuli such as PAMPs, cytokines/chemokines and hormones to regulate cell survival, proliferation, and differentiation. This pathway plays a pivotal role in the activation phenotype of macrophages [for a thorough review, see Vergadi et al. (85)]. PI3K activates downstream kinase AKT that may exist as three different isoforms, namely AKT1, AKT2, and AKT3. AKT signaling is considered to be an activation dampening signal that controls NO and inflammatory cytokine production after TLR signaling (86–88) and promotes anti-inflammatory cytokines such as IL-10 (89, 90). However, AKT signaling is also required for normal M1 responses (91–93). The AKT1 and AKT2 isoforms play opposing roles in macrophage polarization. AKT1 KO macrophages show enhanced iNOS and IL-12 production and bacterial clearance (91, 94, 95). These effects were mediated by induction of the pro-M1 factor miR-155 (96, 97) that suppresses the target CCAAT/enhancer-binding protein beta CEBPβ (91, 95), a pro-M2 factor (98). In contrast, AKT2 deficiency has the opposite effect, resulting in macrophages that express CEBPβ and signature M2 markers such as arginase-1, YM1, REMLα, and the regulatory cytokine IL-10 (91, 99). AKT2 KO macrophages appear to adopt this phenotype *via* high levels of miR-146 (93), which has been associated with dampening of inflammatory responses *via* targeting of IRAK1, TRAF6, and IRF5 leading to suppression of TLR signaling in macrophages and microglia (100–103). Consistent with AKT signaling dampening inflammation, *Akt3* gene deficient mice suffered more severe disease in the murine EAE model of MS, an effect mediated by both peripheral macrophages and microglia (104).

Downstream, the PI3K/AKT pathway regulates cellular metabolism *via* the tuberous sclerosis (TSC)/mammalian target of rapamycin (mTOR) pathway. This is interesting in light of the prominent role metabolism plays in determining macrophage polarization (see metabolism section below). Some data suggest that mTOR signaling inhibits M1 and promotes M2 polarization (105–108). In contrast, other results are more consistent with mTOR signaling promoting M1 polarization (105, 106, 109–112). However, further clarity on the precise roles of TSC and *mTORC1* and *mTORC2* gene isoforms on metabolism and phenotype is required.

In summary, AKT signaling relays diverse extracellular signals to engage the metabolic regulator mTOR pathway. While AKT signaling can activate inflammatory responses, it is essential for promoting a dampening response, thereby promoting resolution.

Notch

Notch signaling controls embryonic development and differentiation in multiple tissues and organs. Notch receptors NOTCH 1-4 are expressed on the cell surface, where they bind Jagged

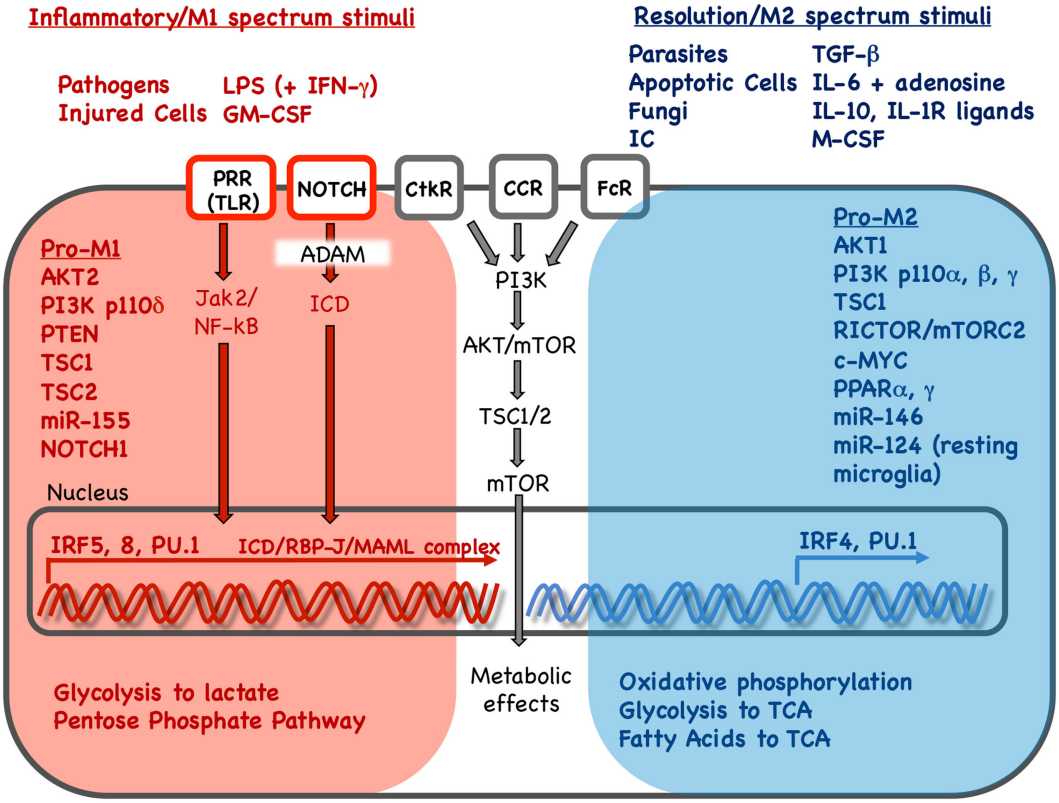

FIGURE 1 | Key stimuli and molecular pathways in inflammatory vs. resolution phenotype in mononuclear phagocytes. Inflammatory and resolution macrophage phenotype results as external stimuli are integrated *via* signaling pathways to drive phenotype-supporting transcriptional programs and cellular metabolism. Red and blue color indicates pathways, stimuli, transcription factors and metabolic processes associated with inflammatory or resolution phenotype, respectively. Inflammatory phenotype is induced or promoted by pathogens, injured cells and *in vitro* stimuli. In contrast, resolution spectrum phenotypes are induced or promoted by parasites, fungi, apoptotic cells, immune complexes and other cytokine/growth factor stimuli. Pathogen or injury signals sensed *via* pathogen-recognition receptors (PRR) such as Toll-like receptors (TLR) result in Janus activated kinase (Jak)2 and nuclear factor kappa B (NF-κB) activation. Signals received *via* Notch receptors, cytokine receptor (CtkR), chemokine receptor (CCR), and Fc receptor (FcR) stimulation are also integrated, defining gene expression and downstream metabolic reprogramming. Interferon regulatory factors (IRFs) 5 and 8 promote inflammatory gene expression while IRF 4 promotes resolution phenotype genes. Gene expression promotes changes in nutrient uptake and metabolic pathways that support inflammatory or resolution macrophage phenotype. LPS, lipopolysaccharide; IFN-γ: interferon-γ; GM-CSF, granulocyte monocyte colony stimulation factor; IC, immune complexes; TGF-β, transforming growth factor-β; IL, interleukin; IL-1R, interleukin 1 receptor; M-CSF, monocyte colony stimulation factor; PI3K, phosphoinositide 3 kinase; AKT, serine threonine kinase; mTOR, mammalian target of rapamycin; PTEN, phosphatase and tensin homolog; TSC, tuberous sclerosis complex; c-MYC, PPAR, peroxisome proliferator activated receptor; ADAM, A disintegrin and metalloproteinase; RBP-J, recombination signal binding protein for immunoglobulin kappa J region; MAML, mastermind-like; Rictor, rapamycin-insensitive companion of mTOR; TCA, tricarboxylic acid/Krebs cycle.

(JAGGED1, JAGGED2) or Delta-like (DLL1, 3, 4) family ligands on neighboring cells. Binding triggers A disintegrin and metalloproteinase (ADAM)/γ-secretase-catalyzed release of the Notch receptors intracellular domain (ICD), allowing ICD translocation to the nucleus, where it heterodimerizes with recombination signal binding protein for immunoglobulin kappa J region (RBP-J). While RBP-J normally acts as a corepressor recruiter, the ICD/RBP-J complex promotes gene expression *via* recruitment of mastermind-like coactivator (MAML) (113, 114). In murine macrophages, increased expression of NOTCH1, NOTCH2, and Notch ligands DLL4 and JAGGED1 has been observed in response to inflammatory cues such as LPS, IFN-γ, or IL-1β (115, 116). NOTCH/RBP-J signaling in macrophages results in enhanced NF-κB signaling and induction of pro-M1 transcription factors IRF1 and IRF8 that in turn drive expression of multiple classical activation genes (115–121). Accordingly, reduced levels of inflammatory cytokines IL-6, IL-12, and IFN-γ are observed in response to LPS + IFN-γ in macrophages deficient in Notch1 or treated with the γ-secretase inhibitor DAPT (122, 123). Notch signaling also increases M1 phenotype by modulating glucose flux to the tricarboxylic acid (TCA) cycle, respiratory chain components and reactive oxygen species (ROS) generation (124). Microglial responses are similarly impacted by NOTCH signaling (125–127). Macrophage-specific *Notch* gene deficiency or γ-secretase inhibitor DAPT treatment suppress clinical disease in *in vivo* disease models, including EAE (126, 128, 129). Overall, NOTCH signaling appears to have a pivotal role in the development of pathogenic macrophage responses and therapeutic strategies that target NOTCH signaling may be beneficial in inflammatory diseases, including MS.

Peroxisome Proliferator Activated Receptors

Peroxisome proliferator activated receptors (PPARs) are nuclear hormone receptors that act as transcription factors and play important roles in development, differentiation and metabolic regulation (130). PPAR ligands include fatty acids, prostaglandins (PG) such as PG J2 and leukotrienes (LT) such as LT B4 (131). PPARs heterodimerize with the retinoid X receptor (RXR) and bind DNA, modulating target gene transcription. There are three PPAR receptors, PPAR α, β/δ, and γ. Stimulation of macrophages with M2 stimuli such as IL-4 and IL-13 induces PPARγ and PPARβ/δ, which are necessary to stabilize M2 phenotype (132, 133).

PPARα activation has been linked to anti-inflammatory innate immune responses in macrophages. PPARα agonists have therapeutic activity in several inflammatory disease models [reviewed in Ref. (134), including EAE (135)]. Similarly, PPARα deficient mice suffer from worsened EAE disease (136). PPARα activation has also been shown to promote regulatory macrophages and mediate microbiota/gut homeostasis (137).

PPARγ activation has long been known to promote M2 polarization and suppress inflammatory cytokines in mouse and human macrophages (138–140). Activation of PPARγ with the flavonoid apigenin suppresses M1 macrophage inflammatory cytokine IL-1β and iNOS expression and promotes expression of alternatively activated phenotype markers by modulating NF-κB signaling (141). Similar suppression of LPS-induced inflammatory microglia has been reported *via* increased PPARγ signals (142). Accordingly PPARγ agonists suppress CNS neuroinflammation in the EAE model (143).

PPARβ/delta is thought to play an anti-inflammatory role, although immune activating effects have also been reported. For example, induction of M2 polarization by IL-4 and IL-13 is dependent on PPARβ/δ (144, 145). In addition, PPARβ/δ agonists suppress intestinal inflammation and EAE (146–148). However, studies in human monocyte-derived macrophages have shown that PPARβ/δ agonists both suppress inflammatory cytokines and suppress cytotoxic T cell inhibitory molecules PD-1L and IDO (149).

In summary, PPAR nuclear receptors are activated by resolution phase lipid mediators, promoting CNS macrophage and microglia phenotype switching toward resolution. Therefore, PPARs stand out as potential therapeutic targets in neuroinflammatory disease.

c-Myc

c-Myc is a transcription factor that modulates cellular survival and proliferation and metabolism, with important roles in angiogenesis, tumorigenesis, and immune responses (71, 150). c-MYC was found first to be induced by M2 stimuli such as IL-4 and IL-13 (71). In human macrophages, c-MYC translocates to the nucleus and controls the expression of half of the M2-associated genes (71). Human M2 markers SCARB, ALOX15, and MRC1 are directly promoted by c-MYC while others are indirectly induced (71). C-MYC also promotes STAT6 and PPAR-γ expression, further stabilizing M2 phenotype (71). In mice, c-Myc has also been found in human tumor-associated macrophages (TAM) (151) and transcriptional profiling of murine BMDM has demonstrated that c-Myc is also a selective marker of murine M2 macrophages (47). c-Myc expression correlated with detectable Egr2 protein, specifically labeling M(IL-4) but not M (LPS + IFN-γ) BMDM macrophages (47).

The exact role of c-MYC in M2 macrophages is not entirely clear. c-MYC may influence macrophage proliferation, which is consistent with the loss of proliferation observed during M1 macrophage stimulation (152). A pivotal role of c-MYC may be to metabolically program the macrophage. While HIF-1α induction in M1 macrophages promotes use of glucose *via* aerobic glycolysis to yield lactate and produce ROS, c-MYC expression in M2 macrophages may allow glycolytic activity necessary for M2 differentiation (153), providing additional sources of fuel for TCA cycle/oxidative phosphorylation (152). To summarize, c-MYC is gaining recognition as a mouse to human-conserved pro-M2 transcription factor. While much research is needed to understand c-MYC's mechanistic actions, its connections to metabolic regulation of phenotype provide an intriguing area for exploration.

Interferon Regulatory Factors

Interferon regulatory factors are transcription factors that are activated in response to cytokines, *via* JAK/STAT signaling, and/or PAMPS and play important roles in innate and adaptive immunity. There are nine IRF family members, named IRF1-9 (154), which modulate macrophage phenotype. Our current understanding is that IRF1, 5 and 8 promote classical activation while IRF4 promotes alternative activation (155).

IRF5 is strongly induced by LPS and IFN-γ and GM-CSF stimuli and plays a prominent role in M1 activation. IRF5 interacts with RelA to bind target gene loci (156), resulting in enhanced IL-12 and IL-23 (157). IRF5 also promotes M1 polarization by association to MyD88 (158). IRF5 variants have been linked to MS (159), possibly *via* enhanced inflammatory activation. Suppression of IRF5 in EAE, *via* inhibition of the Aurora Kinase A, reduced inflammatory cytokines and improved clinical disease (160). Another IRF, IRF1, also contributes to M1 phenotype *via* induction of iNOS and IL-12 (161). IFN-γ stimulation of macrophages induces Batf2, which was shown be an M1-specific factor than interacts with IRF1 to induce Nos2, Tnf-α and Ccl5 (162). IRF8 contributes to M1 phenotype by activating IL-12 transcription in cooperation with IRF1 (163). The clinical relevance of IRF8 is highlighted by the link between IRF8 variants and MS (164, 165). Mice with a myeloid-specific deletion of IRF8 are resistant to EAE. IRF8 activates microglia and drives an IL-12 and IL-23 rich environment that promotes Th1 and Th17 responses (166). In contrast, IRF4 is instead a major mediator of M2 polarization (167). IRF4 inhibits pathogen sensing *via* suppression of MyD88 signaling (167, 168) and collaborates with histone deacetylase Jumanji D3 (Jmjd3) to promote expression of M2 genes Arg1, CD206, Ym1, and Fizz1.

Overall, IRFs essentially link cytokine and PAMP extracellular stimuli to signaling that enhances (IRF1, 5, 8) or suppresses (IRF4) inflammatory transcriptional programs. The association of IRFs to MS risk and EAE disease by impacting macrophages

and microglia highlight the importance of IRFs and their potential as therapeutic targets.

EMERGING PATHWAYS

MicroRNA

MicroRNA are small (~22 nucleotides) RNAs that are regulated in response to inflammatory signals, modulating macrophage and microglia activation and phenotype. The biogenesis of miRNA starts with transcription of a primary (pri-miRNA) transcript that undergoes several processing steps (169). The first involves Drosha/DCGR8 complex cleavage to generate a double-stranded hairpin precursor termed pre-miRNA. This is followed by pre-miRNA export to the cytoplasm, where Dicer eliminates the hairpin yielding a miRNA–miRNA duplex. One miRNA strand is then loaded onto the RISC complex for binding to target mRNA transcript. miRNA generally suppresses target gene expression *via* either induction of mRNA degradation or inhibition of translation (170).

In the context of inflammatory stimuli, miRNA modulate their expression and macrophage polarization (see **Table 2**). The importance of miRNA in macrophage/microglia polarization in now well documented. miRNA such as miR-155, miR-146, miR-101, miR-21 and let-7 family are induced in response to inflammatory stimuli while miR-223, miR-125b, and the miR-23/27a/24-2 cluster are instead downregulated. Among these miRNAs, miR-155 stands out as a miRNA necessary for inflammatory phenotype. miR-146 is instead induced by inflammatory stimuli to dampen the inflammatory response. These two miRNAs and the pathways they control are therefore discussed in detail below, together with a summary of the contributions of other miRNAs to pathways that modulate macrophage and microglia phenotype.

TABLE 2 | Summary of miRNAs linked with M1 or M2 phenotype.

M1 stimulated	
miRNAs with proinflammatory effects	
microRNA	**Modulated Pathways**
miR-92	*Mkkγ*
miR-101	*Mkp1*
miR-105	*Tlr2*
miR-155	*Inpp5d/SHIP-1, Cebpb, Creb, Bcl6, Sfp1, IL-13Ra, SOCS1 and Sfp11, MafB*, and *Tspan14*
miR-223	*Pknox1*
miRNAs with anti-inflammatory effects	
microRNA	**Modulated Pathways**
miR-21	*NFkB, Il6, Tnfa, Tlr4*, and *Tlr8*
miR-101	*Dusp1*
miR-125b	*Irf4*
miR-146	*Irak1, Traf6, Irf5, Tlr4*, and *Stat1*
miR-223	*Cebpb, Rasa1*, and *Nfat5*
M2 stimulated	
miRNAs with proinflammatory effects	
microRNA	**Modulated Pathways**
miR-26	*Atf2* and *Tnf*
miR-27	*Trc4*, *Irak4*, *Il6*, *Il1β*, *Tnfα*, *Nos*
miR-let7b	*Tlr4*, *Tnfα*
miR-let7i	*Tlr4*
miRNAs with anti-inflammatory effects	
microRNA	**Modulated Pathways**
miR-23/27a/24-2	*Jak1/Stat6*, *Irf/Pparγ*, *Tlr4*, and *Irak4*
miR-21	*Arg1*, *Il4α*, *Mrc1*, and *Pge2*
miR-146	*Irak1*, *Traf6*, *Irf5*, *Tlr4*, and *Stat1*
miR-181	*Il1a*, *Il6*

miR-155 is robustly induced in CNS inflammatory conditions such as MS and spinal cord injury (171–174). Several cells in the CNS, including macrophages (175), microglia (176), astrocytes (177), and neurons (178) may express miR-155. miR-155 is the most highly upregulated miRNA after exposure to a range of inflammatory stimuli including M [LPS + (IFN-γ)] conditions in both murine/human macrophages (96, 173, 179–183) and microglia (173, 176, 184). In contrast, exposure to alternatively activating stimuli such as IL-4 does not induce miR-155 (96, 185). The quick and swift induction of miR-155 suggests that miR-155 plays a crucial role in determining the classically activated macrophage phenotype. In support of this hypothesis, miR-155 delivery into macrophages or microglia *via* exosomes enhances inflammatory gene expression, including IL-6 and IL-12 (186). miR-155 in microglia also modulates phenotype *via* suppression of SOCS-1 and enhancement of NO and cytokine production (176). So, what are the global effects of miR-155 on M1 phenotype? Transcriptional profiling in miR-155 KO macrophages exposed to M(LPS + IFN-γ) conditions reveals that approximately half of the M(LPS + IFN-γ) signature is miR-155 dependent (96). These results indicate that miR-155 is required for full expression of inflammatory macrophage signature. Among the most impacted genes, inflammatory cytokines such as IL-1β, IL-6 and IL-12, inflammatory enzymes such as iNOS and costimulatory molecules such as CD86 and CD40 and adhesion and migration molecules such as CD49E and CCR7 stand out (96, 97). These data are consistent with clinical improvement of EAE, SCI and stroke in animals deficient in miR-155 or treated with miR-155 antisense oligonucleotide inhibitors (171, 187). Since miRNAs typically suppress targets rather than promote them, the inflammatory gene activation effects of miR-155 are expected to be mediated by suppression of deactivating genes. Validated miR-155 targets include transcripts of the *Inpp5d, Cebpβ*, *Creb*, *Bcl6*, *Sfp1*, *IL-13Rα*, *Socs1*, *Sfp11*, *MafB*, and *Tspan14* genes. We found that the transcripts of target genes *Inpp5d*, *Ptprj*, *MafB* and *Tspan14* inversely correlated with miR-155 (96). Targeting of *Inpp5d* by miR-155 promotes AKT signaling (188) in murine macrophages. In addition, CEPBβ has been shown to promote alternatively activated genes IL-10, IL-13Ra, arginase-1, RELMα (98) and targeting by miR-155 may suppress M2 phenotype. This is in contrast to the later finding that CEBPβ targeting by miR-223 is essential to prevent inflammatory macrophage development and colitis (189). MAF suppresses IL-12 and promotes IL-10 production in macrophages (190, 191) and its inactivation by

miR-155 may be required to initiate the inflammatory gene expression program.

Although miR-146a/b are coinduced with miR-155 in response to M1 stimuli (192), they have opposite effects. While miR-155's role is to release the brake on inflammation, miR-146 instead sets off a series of events that will eventually dampen inflammation. miR-146 targeting of TRAF6, IRAK1, TLR4, and STAT1 appear to mediate some of these effects by limiting responsiveness to inflammatory stimuli (100–102). Similar results have been observed in microglia, where miR-146 has been shown to promote M2 phenotype by targeting IRAK1/TRAF6 (103).

Other miRNAs modulated with macrophage/microglia phenotype include miR-124, miR-125b, miR-223, miR-101, miR-21, the let-7 family and the miR-23a/27a/24-2 cluster. Effects on phenotype appear to be achieved through targeting to JAK/STAT, NF-kB, or MAPK pathways and CEBP, PPAR, or IRF family transcription factors as further described below or in **Table 2**.

The CEBP family of transcription factors is targeted by miR-124, miR-223, and let-7 family miRNAs in macrophages and microglia. miR-124 and miR-223 target CEBPβ, which promotes macrophage development (98) and both inflammatory (193–196) and alternatively activating cytokines (197, 198). miRNA-124 is expressed in microglia, promoting a resting phenotype, but not in peripheral origin macrophages/monocytes (199). miR-124 treatment suppresses microglia and macrophage activation, T cell infiltration and clinical disease in EAE and other CNS inflammatory models, suggesting it may beneficial in MS (199, 200). miR-223 maintains a deactivated state through targeting of CEBPβ (189). miR-let-7c is associated with less inflammatory GM-CSF induced macrophages, where it suppresses the pro-inflammatory TF CEPBδ (201). miR-let-7i (202) and miR-let-7b (203) have also been shown to dampen inflammation by suppression of TLR4 expression.

Many miRNAs modulate macrophage phenotype by dampening or promoting the NF-kB pathway that proinflammatory stimuli induce. For example, miR-125b promotes activated microglia phenotype by suppressing A20/NF-κB signaling (204). miR-let-7f also targets the NF-κB negative regulator A20, resulting in enhanced IL-1β and TNF-α (205). In addition, let-7b also acts as a TLR7 agonist in microglia, activating TLR signaling and downstream NF-kB activity and leading to inflammatory microglia and neurodegeneration (206). Consistent with this, let-7b correlates with TNF-α production in tumor-associated macrophages (207).

In summary, miRNAs are now established as important regulators of macrophage and microglia that modulate neuroinflammation and neurodegeneration. miR-155 and miR-146 have robust pro- and anti-inflammatory roles, respectively, that may be therapeutically harnessed in MS and other neuroinflammatory diseases.

Metabolism

Metabolism is taking center stage in our understanding of pathways that modulate macrophage phenotype. While it was understood that inflammatory macrophages necessitate metabolic adaptations to offset high energy requirements, the realization that metabolism in fact *determines* inflammatory or regulatory phenotype is possibly a paradigm shift. Since metabolic pathway choice depends on enzyme activity, it provides interesting new (or repurposed) therapeutic strategies for inflammatory disease, further discussed in section 8. For a summary of how metabolic pathways differ in M1 and M2 macrophages, refer to **Figure 2**.

Differences in amino acid metabolism have long been observed among macrophage phenotypes (208, 209). While M1 macrophages upregulate iNOS to convert arginine to NO for microbial killing, M2 macrophages induce arginase-1 and catabolize arginine to produce polyamines and proline for collagen biosynthesis. Additional differences in ATP generation (glycolysis vs. mitochondrial oxidative phosphorylation), pentose phosphate pathway activity, and TCA use have recently been demonstrated (210).

- ATP generation. Most tissues, including M2 macrophages, use mitochondrial oxidative phosphorylation (34 ATP/glucose or 129/palmitic acid) as a source of energy. In contrast, M1 macrophages rely on aerobic glycolysis to lactate (2 ATP/glucose) for energy generation (211). Aerobic glycolysis is also known as Warburg metabolism and this phenotype has also been observed in cancer cells (212).

Why a lower ATP output pathway would be chosen by M1 macrophages is intriguing. Glycolysis may provide rapid ATP production. However, new evidence that NO production in M1 macrophages strongly inhibits oxidative phosphorylation by impairing the electron transport chain (213) provides an alternative explanation for the need to use an alternative pathway for ATP generation.

While M1 macrophages have increased glycolytic activity as compared to M2 macrophages, M2 macrophages also depend to some extent on access to glucose and its oxidation *via* glycolysis. Although the TCA cycle in M2 macrophages was thought to be mostly fueled by fatty acids (210), recent work by has shown that an active glycolysis pathway is essential for TCA/oxidative phosphorylation and M2 phenotype (153).

- Pentose phosphate pathway (PPP). The PPP directs some glucose-6P away from the glycolysis pathway and into generation of ribose-6P and derivatives. This pathway yields nucleotides to support DNA replication and RNA transcription and NADPH for ROS and NO generation. In addition to increased glycolysis, high PPP activity is characteristic of M1 macrophages (214).
- Krebs/TCA cycle. M2 macrophages rely almost exclusively on ATP generation *via* oxidative phosphorylation coupled to an intact TCA cycle (213, 215, 216). In contrast, in M1 macrophages the TCA cycle is "broken" at two steps: citrate to α-ketoglutarate and succinate to fumarate (217, 218). Reduced isocitrate dehydrogenase activity leads to citrate accumulation in M1 macrophages. Citrate supports M1 phenotype by promoting FAS and membrane biosynthesis, prostaglandin and itaconate production. Itaconate inhibits succinate dehydrogenase causing the second break in the cycle and succinate accumulation (219). Succinate stabilizes HIF-1α and promotes IL-1β production in LPS stimulated macrophages (217, 218). The second break in the TCA cycle is linked to a reversal in

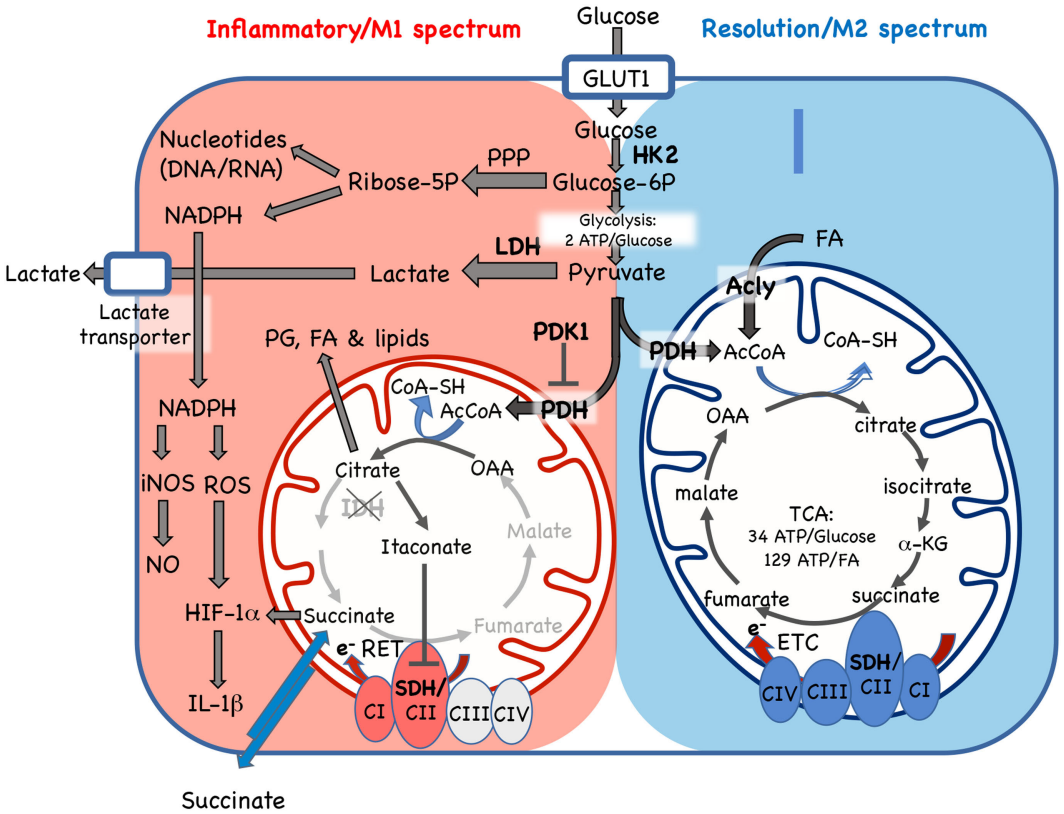

FIGURE 2 | Cellular metabolic pathways driving mononuclear phagocyte inflammatory vs. resolution phenotype. Glucose is essential for both inflammatory and resolution macrophage phenotypes. In inflammatory macrophages, glucose is largely processed to yield lactate *via* aerobic glycolysis. Another major pathway in M1 spectrum macrophages is conversion to ribose-5P *via* the pentose phosphate pathway (PPP) for synthesis of nucleotides and NADPH, which supports nitric oxide (NO), reactive oxygen species (ROS), and IL-1β production. In resolution macrophages, the major fate of glucose is the TCA/Krebs cycle *via* pyruvate dehydrogenase (PDH)-catalyzed conversion of pyruvate to AcetylCoA (AcCoA). The TCA cycle in resolution macrophages is also fed by fatty acids (FA) *via* the ATP-citrate lyase (Acly) enzyme and promotes forward electron transport chain (ETC), from C1 to CIV, for ATP generation. In contrast, while some glucose enters the mitochondria in inflammatory macrophages, where it is converted to citrate, the TCA cycle is broken, with stops at the isocitrate dehydrogenase (IDH) and succinate dehydrogenase (SHD) steps. Citrate accumulation results in itaconate production, which inhibits SDH, and also promotes prostaglandin (PG), lipid and FA synthesis. In inflammatory macrophages, these blocks promote reverse electron transport chain (RET) from CII to CI. GLUT1, glucose transporter 1/SLC2A1; HK2, hexokinase 2; Glucose-6P, glucose-6-phosphate; LDH, lactose dehydrogenase; PHK1, pyruvate dehydrogenase kinase 1; AcCoa, acetyl coenzyme A; TCA, tricarboxylic acid/ Krebs cycle; a-KG, a-ketoglutarate; CI-IV, ETC complexes I–IV, SDH, succinate dehydrogenase; OAA, oxaloacetate; e^-, electrons; RET, reverse electron transport chain; iNOS, inducible nitric oxide synthase; HIF-1α, hypoxia inducible factor-1α. Enzymes are indicated by bold font.

electron transport chain direction that fuels an increase in mitochondrial membrane potential and supports classic M1 NO and ROS generation (217).

In summary, M1 and M2 macrophages use opposing metabolic pathways to fulfill energy and biosynthetic requirements. M1 macrophages favor glycolysis to lactate and PPP pathways to provide energy and support NO and ROS generation. M2 macrophages instead rely on TCA cycle for ATP generation. This metabolic dichotomy is not a consequence of M1 or M2 phenotype, but rather a requirement for either phenotype, therefore providing exciting opportunities for therapeutic targeting.

Receptor-Tyrosine Kinases

Receptor-tyrosine kinases have been proposed to fine-tune macrophage function in immunity and tissue homeostasis. Macrophages are known to express RTKs within three families of RTKs, namely platelet-derived growth factor receptor (PDGFR), the AXL/TYRO3/MERTK family, and the RON superfamily. Colony stimulation factor receptor 1 (CSF1R) is involved in macrophage development and is a member of the PDGFR superfamily (220). The PDGFR family receptors are characterized by 5 Immunoglobulin (Ig)-family domains and a kinase domain. The TAM (TYRO3/AXL/MERTK) family is instead characterized by two Ig-like domains, two fibronectin III repeats and a Kinase domain (221, 222). Finally, the human RON receptor (STK in mouse) is a member of the MET family of RTKs (223, 224). Ligation of RTKs to ligands, such as M-CSF to CSF1R, apoptotic cell phosphatidyl serine (PtdSer) *via* grown arrest specific 6 (Gas6) and Protein S bridging to TAM family RTKs and macrophage-stimulating protein (MSP) to STK results in activation of kinase activity (225–227).

The active form of STK ligand MSP is generated *via* the coagulation cascade (228) and has a crucial role in the response

of macrophages to inflammatory cytokines and LPS. MSP dampens NO and PGE2 production *via* suppression of iNOS and COX-2 expression (229–231). TAM receptors mediate apoptotic cell removal after PtdSer is recognized *via* Gas6 or Protein S, activating MerTK and reducing TNF-α and LPS responsiveness (232). NO activity, IL-12 production and MHCII expression are also controlled by MSP: MSP exposure prior to LPS + IFN-γ activation inhibits these signature M1 factors *via* arginase-1 induction (233). Consistent with the proresolving role of RTKs, macrophages from *Tyro3*, *MerTK*, and *Axl* triple KO mice display enhanced IL-12, MHCII, and costimulatory molecules in response to LPS (234).

The AXL RTK is also induced in mouse and human macrophages by type I IFNs and TLR3 stimulation (235). This induction may signal enhanced apoptotic cell removal needs during inflammation. The Rothlin group added a relevant layer to the physiologic role of TAM RTKs AXL and MERTK in resolution of immune responses to infection and promotion of tissue repair (60). They found that including apoptotic cell ligands for TAM RTKs strongly enhanced the expression of anti-inflammatory and tissue repair genes, including RELMα, CHI3L3, FN1, and EAR2 in response M2 (IL-4) stimuli (60) *in vitro*. Such signaling was essential to dampen inflammation and allow tissue repair in thioglycollate and helminth infection models. Their results implicate that apoptotic cell sensing by AXL and MERTK in the presence of IL-4 responses drives anti-inflammatory and tissue repair programs in macrophages. It is interesting to speculate whether the failure of most interventions to enhance M2 phenotype during injury (236, 237) may stem from deficiencies in these pathways. If so, small molecule-mediated stimulation of these pathways during chronic CNS inflammation may be a promising therapeutic strategy to promote proregenerative macrophages/microglia.

In summary, RTK activity is largely stimulated by resolution mediators, such as MSP and apoptotic neutrophil PtdlSer in late stages of acute inflammation. These signals effectively suppress inflammatory responses and promote and amplify a resolution macrophage phenotype switch. These findings highlight a physiologically relevant pathway for inflammation resolution that may be therapeutically harnessed in neuroinflammatory disease.

THERAPEUTIC STRATEGIES FOR MACROPHAGE/MICROGLIA PHENOTYPE REPROGRAMMING

Increased understanding of the molecular pathways that promote inflammatory and resolution phenotypes in macrophages and microglia provides therapeutic targets for inflammatory diseases, including the autoimmune disease MS. Due to the increasing importance of metabolic reprogramming in macrophage phenotype, we will focus our discussion on the current understanding of how available metabolic reprogramming drugs may impact macrophage phenotype and MS.

Dichloroacetate (DCA) is an inhibitor of PDK1, a kinase that in turn suppresses PDH. DCA treatment therefore increased PDH activity, shifting cellular metabolism toward the TCA cycle/oxidative phosphorylation and promoting a proregenerative M2 resolution phenotype (238). Consistent with a shift from M1 to M2 phenotype in macrophages and microglia, treatment with DCA suppressed clinical disease scores and T cell infiltration in the EAE model of MS (239).

Dimethylfumarate (DMF) is an approved oral drug for MS treatment that has been shown to efficiently reduce relapse rate and disease progression (240). DMF is thought to exert its therapeutic effects *via* activation of the Nrf2 pathway and induction of the antioxidant response. Could DMF's metabolite monomethyl fumarate enter the TCA cycle at the fumarate step, thereby modulating oxidative phosphorylation? Consistent with this scenario, increased TCA cycle intermediates malate, fumarate and succinate are observed in DMF-treated oligodendrocytes (241). However, it is unknown whether similar effects occur in macrophages and microglia. Microglia and myeloid cell pretreatment with DMF does however reduce NO and inflammatory cytokine production (242, 243), although these effects have not been recapitulated in DMF-treated EAE mice (244). Similarly, increased superoxide generation has been observed in fumarate-treated monocytes (245), indicating that DMF's effects may depend on timing and context.

Many of the current drugs targeting metabolic pathways are currently approved for type II diabetes and metabolic syndrome therapy. One of the first drugs for type II diabetes, the biguanide family metformin, inhibits AMPK and complex I of the electron transport chain (ETC) (246). Treatment of LPS-activated macrophages with metformin suppresses NO and IL-1β production while increasing IL-10 (247). This effect was due to inhibition of complex I, which is necessary for respiratory electron chain in M1 macrophages (247). *In vivo* metformin treatment reduced inflammatory cytokine production, reduced Th17 responses and enhanced Tregs, overall ameliorating EAE disease (248, 249). The common use of metformin for metabolic syndrome has allowed to test whether it is beneficial in MS. In an open-label study of 50 obese MS patients, the 20 patients who received metformin had decreased new or enlarging T2 and gadolinium-enhancing lesions (250, 251). These clinical effects were accompanied by significant decreases in inflammatory Th1 and Th17 cells but significant increases in Tregs in metformin-treated patients (250).

Pioglitazone and rosiglitazone are antidiabetic drugs from the thiazolidinedione family that act as full agonists of PPARγ, modulating multiple metabolic processes, particularly lipid and glucose metabolism (130, 252). A smaller number of patients in the metformin study above were treated with pioglitazone and similarly benefited from reduced lesions and shifts from inflammatory to regulatory T cell responses (250). These clinical effects are consistent with previously reported suppression of microglial activation and clinical disease in the EAE model (253, 254).

CONCLUDING REMARKS

Recent studies have provided us with a more thorough and insightful understanding about how steady-state and acute environmental signals are integrated by macrophages and their microglial counterparts to maintain optimal neurologic function, eliminate infection and resolve injury or inflammation.

Besides PRR-induced Jak2/NF-κB signaling, Notch receptor signaling and PI3K/AKT2/miR-155 signaling contributes to M1 phenotype. In contrast, PI3K/AKT1/miR-146 and apoptotic cell-induced RTK signaling promotes M2 phenotype. These effects are achieved through IRF-mediated control of gene expression, leading to changes in how cellular nutrients are used *via* metabolic pathways. M1 macrophages turn to aerobic glycolysis/Warburg metabolism and high PPP activity for energy production and simultaneously promote NO, ROS, and IL-1β expression. Interestingly, NO inhibits oxidative phosphorylation, which may explain the need for TCA-independent sources of energy in M1 macrophages. In contrast, M2 macrophage polarization and function is entirely dependent on TCA/oxidative phosphorylation, although glucose is also essential for M2 phenotype. Links between classic pathways known to affect macrophage phenotype and metabolism are starting to emerge. The PI3K/AKT pathway activates mTOR signaling, a major regulator of glucose metabolic pathways in the cell. Similarly, PPARγ is another major regulator of macrophage phenotype and lipid/glucose metabolism. Finally, c-MYC is an important mediator of M2 phenotype in murine and human macrophages that modulates glucose fuel utilization. The requirement of active metabolic pathways for specific macrophage phenotypes constitutes a paradigm shift in the field, away from a mere supporting role. Perhaps more importantly, it provides an opportunity for therapeutic modulation at the step where signaling pathways converge to determine phenotype. Novel and repurposed metabolic reprogramming drugs may provide promising alternative therapeutic strategies in MS and other neuroinflammatory disorders. So far, a study has shown excellent clinical MS responsiveness using metformin and pioglitazone in obese MS patients. Further studies are required to evaluate the efficacy of these treatments in the general MS population and understand how they impact macrophages and microglia phenotype. The field is ripe to address these questions and exciting basic knowledge and therapeutic opportunities lie ahead.

AUTHOR CONTRIBUTIONS

All authors: conception of idea, review of the literature, manuscript writing and editing.

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Modulation of P2X7 Receptor during Inflammation in Multiple Sclerosis

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Multiple sclerosis (MS) is characterized by macrophage accumulation and inflammatory infiltrates into the CNS contributing to demyelination. Because purinergic P2X7 receptor (P2X7R) is known to be abundantly expressed on cells of the hematopoietic lineage and of the nervous system, we further investigated its phenotypic expression in MS and experimental autoimmune encephalomyelitis conditions. By quantitative reverse transcription polymerase chain reaction and flow cytometry, we analyzed the P2X7R expression in human mononuclear cells of peripheral blood from stable and acute relapsing-remitting MS phases. Human monocytes were also challenged *in vitro* with pro-inflammatory stimuli such as the lipopolysaccharide, or the P2X7R preferential agonist 2′(3′)-O-(4 Benzoylbenzoyl)adenosine 5′-triphosphate, before evaluating P2X7R protein expression. Finally, by immunohistochemistry and immunofluorescence confocal analysis, we investigated the P2X7R expression in frontal cortex from secondary progressive MS cases. We demonstrated that P2X7R is present and inhibited on peripheral monocytes isolated from MS donors during the acute phase of the disease, moreover it is down-regulated in human monocytes after pro-inflammatory stimulation *in vitro*. P2X7R is instead up-regulated on astrocytes in the parenchyma of frontal cortex from secondary progressive MS patients, concomitantly with monocyte chemoattractant protein-1 chemokine, while totally absent from microglia/macrophages or oligodendrocytes, despite the occurrence of inflammatory conditions. Our results suggest that inhibition of P2X7R on monocytes and up-regulation in astrocytes might contribute to sustain inflammatory mechanisms in MS. By acquiring further knowledge about P2X7R dynamics and identifying P2X7R as a potential marker for the disease, we expect to gain insights into the molecular pathways of MS.

Keywords: astrocytes, monocyte chemoattractant protein-1, monocytes, multiple sclerosis, neuroinflammation, P2X7 receptor

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Abbreviations: BzATP, 2′(3′)-O-(4-Benzoylbenzoyl)adenosine 5′-triphosphate; CD, cluster of differentiation; CFA, complete Freund's adjuvant; EAE, experimental autoimmune encephalomyelitis; GFAP, glial fibrillary acidic protein; HD, healthy donors; LPS, lipopolysaccharide; MBP, myelin basic protein; MHC II, major histocompatibility complex II; MS, multiple sclerosis; NDS, normal donkey serum; PBMCs, peripheral blood mononuclear cells; P2X7R, P2X7 receptor; RT-qPCR, quantitative reverse transcription polymerase chain reaction; SPMS, secondary progressive MS.

INTRODUCTION

Peripheral and central mechanisms provide insights into the cellular basis of neuroinflammation that leads to severe demyelination and neurodegeneration in multiple sclerosis (MS). During both MS and experimental autoimmune encephalomyelitis (EAE), monocyte-derived macrophages are part of the inflammatory CNS infiltrates and accumulate during the disease concomitantly with active demyelination, while CNS-resident microglia-derived macrophages are inert at disease onset and participate to later phases of the disease. Autoreactive myelin-specific T cells then boost inflammation, demyelination and CNS damage, contributing to neurological deficit, and blood–brain barrier dysfunction (1). In addition, astrocytes appear to have a dual role in MS, depending on the disease status and lesion topography, and contributing in both pathogenic alterations and beneficial repair (2–6). In examining those mechanisms that converge in causing inflammatory demyelination, the analysis of frontal cortex constitutes a convenient experimental platform, because profuse lesions in cerebral cortex constitute a significant proportion of MS pathology, and characterize the evolution from a relapsing/remitting early phase into a secondary progressive MS (SPMS) (7–9).

Extracellular purine/pyrimidine nucleotides and nucleosides binding to several different purinergic receptors are among the most diffuse exogenous signals playing important biological functions in the CNS, being responsible for the cell-to-cell communication under normal and pathological conditions (10–14). A member of the purinergic P2X family of ATP-gated ion channels, the P2X7 receptor (P2X7R) (15) is selectively expressed on cells of the hematopoietic lineage (16–19). Moreover, in the nervous system, P2X7R is present on activated microglia (20–22), astrocytes (23–25), oligodendrocytes (26–28), Schwann cells (29), and neurons (30). Despite its wide expression in many cell types participating to MS, only incomplete information is available regarding P2X7-mediated signaling in the disease. For instance, in optic nerve P2X7R expression is augmented in oligodendrocytes and myelin sheaths in MS and EAE before lesion formation, thus contributing to tissue damage; as a consequence, P2X7R blockade prevents oligodendrocyte excitotoxicity and ameliorates EAE (31). P2X7R immunoreactivity is augmented also in activated microglia/macrophages in spinal cord during MS, and extracellular ATP apparently contributes to MS lesion-associated release of interleukin-1β from microglia/macrophages, *via* P2X7R-dependent induction of cyclooxygenase-2 and downstream pathogenic mediators (21). Mice deficient in P2X7R function are more resistant to EAE than wild-type mice, also showing reduced CNS inflammation, axonal damage, and astrocytes activation (32). Furthermore, pharmacological blockade of the receptor remarkably diminishes astrogliosis in rat EAE and alleviates neurological symptoms (24). On the other hand, it was also reported that P2X7R knockdown causes a more severe EAE and that lymphocyte from P2X7R$^{-/-}$ mice proliferate more vigorously *in vitro*, producing reduced levels of IFN-γ and NO, thus suggesting an important role for this receptor in MS lymphocyte homeostasis (33).

The aim of the present work is to further investigate the role of P2X7R in MS pathogenesis, by analyzing its expression in peripheral blood mononuclear cells (PBMCs) from stable and acute phases of relapsing-remitting MS and in human frontal cortex of SPMS.

MATERIALS AND METHODS

Ethical Statement

Blood samples were obtained following acquisition of the study participants' informed consent. The protocol was approved by ethic committees of San Camillo Forlanini Hospital. Patients enrolled were diagnosed with relapsing-remitting form of MS according to 2005-revised McDonald's criteria (34). Frontal cortex tissue was collected postmortem by UK MS Tissue Bank at Imperial College, London, and procedures for retrieval, processing, and storage have gained ethical approval.

Flow Cytometry and Human Monocytes Separation

Peripheral blood mononuclear cells were isolated by a density gradient centrifugation over a Ficoll-Hypaque (Ficoll-Paque PLUS, GE Healthcare) from 20 ml of freshly venous blood from five healthy donors (HD), five relapsing MS patients (MS acute), and five remitting MS patients (MS stable). Cells were stained with pre-titrated Abs, to evaluate the expression of P2X7R within cluster of differentiation 14 (CD14)-positive cells. Briefly, PBMCs (1×10^6) were incubated with P2X7-extracellular epitope antibody (Alomone Labs, Jerusalem, Israel) for 30 min at 4°C. Cells were washed and stained with goat anti-rabbit Alexa Fluor 488-conjugated antibody (Invitrogen, Life Technologies, Monza, MB, Italy), 30 min at 4°C. Cells were washed and stained with anti-CD14 PE (Dako, Aurogene, Rome, Italy) and Live Dead Fixable Aqua Dead Cell Stain Kit (Invitrogen) for 30 min at 4°C.

Monocytes were isolated from PBMCs of HD by using Magnetic Separation with Negative Selection Columns (Miltenyi Biotec, Calderara di Reno, BO, Italy) according to the product manual. Purified monocytes (6×10^6) were cultured in serum-free RPMI 1640 with L-Glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin in 96-well plates.

FACS analysis was performed with FACS CyAn (Beckman Coulter, Pasadena, CA, USA) and with advanced flow cytometry software FlowJo (Tree Star, Ashaland, OR, USA).

Quantitative Reverse Transcription Polymerase Chain Reaction (RT-qPCR)

Human and rat monocytes or snap-frozen tissues were homogenized in TRIzol (Life Technologies) and total RNA was extracted following the manufacturer's instructions. UV spectrophotometric analysis of nucleic acids was performed by Nanodrop spectrophotometer at 260 nm to determine concentration. The 260:280 absorbance ratio was used to assess nucleic acids purity. After DNase treatment (Qiagen, Hilden, Germany), 1 μg of total RNA was subjected to retro-transcription by high-capacity RNA-to-cDNA kit (Applied Biosystem, Life Technologies).

Quantitative polymerase chain reaction was carried out using SYBR green (Applied Biosystem, Life Technologies) incorporation with gene-specific primers (**Table 1**). Relative gene expression was calculated by $\Delta\Delta Ct$ analysis relative to GAPDH.

EAE Rat Model

Female Lewis rats (~160 g, 6 weeks old) were purchased from Charles River (Como, Italy). Animal procedures were performed according to European Guidelines for animal use in research (86/609/CEE) and requirements of Italian laws (D.L. 116/92), according to protocol no. 112/2014B by Italian Ministry of Health. Efforts were made to minimize animal suffering and the number of animals used.

Female rats were deeply anesthetized and injected in each hind paw with 100 µl of a medium containing 0.15 g/ml guinea pig spinal cord tissue in saline (0.9% NaCl) and complete Freund's adjuvant (CFA, Sigma-Aldrich, Milan, Italy), 50% vol/vol, to which 5 mg/ml heat-inactivated *Mycobacterium tuberculosis* (Difco H37Ra) were added. CFA-injected rats were used as control of inflammation.

Starting at 5-day postinjection, all animals were daily weighed, assessed for clinical signs of disease, and graded according to the following described criteria: 0 = no clinical signs; 1 = loss of tail tonus; 2 = weakness in one or both hind legs or mild paresis; 3 = severe paresis or paralysis of both hind legs; 4 = severe paralysis of complete lower part of the body; and 5 = death due to aggressive EAE (35).

Rat and Mouse Monocytes Separation

Female C57BL/6 mice (~25 g, 8 weeks old) were purchased from Charles River (Como, Italy). Animal procedures were performed according to European Guidelines for animal use in research (86/609/CEE) and requirements of Italian laws (D.L. 116/92), according to protocol no. 119/2015PR by Italian Ministry of Health. Efforts were made to minimize animal suffering and the number of animals used.

Female Lewis rats ($n = 4$) and C57BL/6 mice ($n = 3$) were sacrificed by asphyxiation with CO_2 and spleen excised for monocytes purification. CFA ($n = 3$) and EAE ($n = 4$) rats were sacrificed at 15 days postinjection by asphyxiation with CO_2. After spleen harvest, single cell suspension was obtained by mechanical tissue dissociation in RCB buffer (NH4Cl 150 mM, NaHCO3 10 mM, and EDTA 1 mM). Cells were plated (4×10^6/ml) in RPMI, 10% fetal bovine serum, 100 U/ml gentamycin, 100 µg/ml streptomycin, and 100 U/ml penicillin. After 2 h, non-adherent cells were removed and medium enriched with 10 ng/ml rat or mouse macrophage colony-stimulating factor (Sigma-Aldrich) (36). After 1 week, cells were used for RT-qPCR, western blotting, and immunofluorescence analysis.

TABLE 1 | List of primer sequences used in this study.

Primer, F: forward primer, R: reverse primer	Sequence, 5′ to 3′	Type of analysis
Rat P2rx7 F	CTGGTGTCCTGCTGAGGAAG	RT-qPCR
Rat p2rx7 R	CTCGTAGTATAGTTGTGGCCCG	RT-qPCR
Human P2rx7 F	ATACAGTTTCCGTCGCCTTG	RT-qPCR
Human P2rx7 R	AACGGATCCCGAAGACTTTT	RT-qPCR
Rat Il-6 F	GAGGATACCACCCACAACAGACC	RT-qPCR
Rat Il-6 R	CAGTGCATCATCGCTGTTCATACA	RT-qPCR
Rat GAPDH F	GCATCTTCTTGTGCAGTGCC	RT-qPCR
Rat GAPDH R	TACGGCCAAATCCGTTCACA	RT-qPCR
Human GAPDH F	TCTTTTGCGTCGCCAGCCGAG	RT-qPCR
Human GAPDH R	TGACCAGGCGCCCAATACGAC	RT-qPCR

RT-qPCR, quantitative reverse transcription polymerase chain reaction.

In Vitro Treatments

Human monocytes were stimulated without or with lipopolysaccharide (LPS, Sigma-Aldrich) or 2′(3′)-O-(4-Benzoylbenzoyl) adenosine 5′-triphosphate (BzATP, Sigma-Aldrich) for 4 h (T4) and 24 h (T24) at 37°C in a 5% CO_2 environment. After treatments, monocytes were incubated with monoclonal antibody as described above, to evaluate P2X7R expression by FACS analysis. Moreover, monocytes from control rats and mice were stimulated *in vitro* with or without LPS or BzATP for 4 h (T4) or 24 h (T24) and P2X7R expression was analyzed by western blotting.

Human Brain Tissue

Demographic and clinical characteristics of MS cases at the time of tissue collection are reported (**Table 2**). Frontal cortex tissues are from 13 neuropathological confirmed cases of MS, matched for disease courses (all secondary progressive MS, SPMS) but presenting different ages (range 34–80 years), sex, disease durations (range 6–50 years), and causes of death (see **Table 2**). Analysis was performed also on samples from four patients who died by non-neurological diseases. Cerebral hemispheres were fixed with 4% paraformaldehyde for 2 weeks, coronally sliced, and blocked. Individual blocks were cryoprotected in 30% sucrose for 1 week, frozen in isopentane, and stored at −80°C until use.

Immunohistochemistry

Immunohistochemistry was performed as described (37). Human sections (30–40 µm) were pre-incubated for 10 min with 5% H2O2 in 5% methanol in PBS, and for 24–48 h in PBS-0.3% Triton X-100, 2% normal donkey serum (NDS) at 4°C, with goat anti-P2X7 receptor antibody (1:100, peptide YETNKVTRIQSMNY-C from the *N*-terminus of human P2RX7 corresponding to amino acid residues 13-26, MyBioSource, San Diego, CA, USA). Sections were then incubated with biotinylated donkey anti-goat antibodies (Jackson ImmunoResearch Europe Ltd., Suffolk, UK), followed by avidin–biotin–peroxidase reactions (Vectastain, ABC kit, Vector, Burlingame, CA, USA), using 3,3′-diaminobenzidine (Sigma-Aldrich) as a chromogen. Some sections were counterstained with Luxol fast blue, in order to detect lesion types. Sections were mounted on poly-lysine slides and air dried for 24 h. The histological preparations were examined using an Axioskop 2 light microscope (Zeiss). Images were taken with Neurolucida software (MBF Bioscience, USA).

Immunofluorescence

Human sections (30–40 µm) were blocked with 10% NDS in 0.3% Triton X-100 in PBS and incubated with primary antisera/

TABLE 2 | Summary of patients information.

Case	Age (years)	Sex	Clinical diagnosis	Disease duration (years)	Cause of death	DTPI (h)
MS062	49	F	SPMS	19	Respiratory infection	10
MS073	80	F	SPMS	50	Bronchopneumonia	20
MS074	64	F	SPMS	36	Gastrointestinal bleed/obstruction, aspiration pneumonia	7
MS076	49	F	SPMS	18	Chronic renal failure, heart disease	31
MS079	49	F	SPMS	23	Bronchopneumonia, MS	7
MS088	54	F	SPMS	17	Bronchopneumonia	22
MS105	73	M	SPMS	46	Pneumonia	8
MS114	52	F	SPMS	15	Pneumonia, sepsis, pulmonary embolism	12
MS125	76	F	SPMS	31	MS	13
MS128	78	F	SPMS	50	Small bowel obstruction, pneumonia	22
MS136	40	M	SPMS	9	Respiratory infection	10
MS154	34	F	SPMS	11	Pneumonia	12
MS163	45	F	SPMS	6	Urinary tract infection, MS	28

Demographic and clinical characteristics of MS cases at the time of tissue collection are reported.
DTPI, death-tissue preservation interval; SPMS, secondary progressive multiple sclerosis; MS, multiple sclerosis.

TABLE 3 | Antibodies used in this study.

Antigen	Clone	Epitope (aa)	Target	Dilution	Source
CD45	61D3	[a]	Leukocyte	1:100	Dako
CD14 biotinylated	T29/33	[a]	Monocytes	1:100	eBioscience
CD68	EBM11	[a]	Macrophages/microglia	1:100	Dako
CD68	Polyclonal	100–354	Macrophages/microglia	1:200	Santa Cruz
GFAP	5C10	[a]	Astrocytes	1:500	Novusbio
HLA-DP, DQ, DR (MHC II)	CR3/43	[a]	Macrophages/microglia	1:100	Dako
MBP	2	119–131	Mature oligodendrocytes/myelin	1:100	Chemicon
MCP-1	Polyclonal	62–89	CCL2 chemokine	1:20–1:1,000	ThermoFisher Scientific
P2X7-intracellular receptor, N-terminus	Polyclonal	13–26	P2X7 receptor	1:100	MyBioSource
P2X7-extracellular receptor	Polyclonal	136–152	P2X7 receptor	1:500	Alomone
P2X7-intracellular receptor, C-terminus	Polyclonal	576–595	P2X7 receptor	1:500	Alomone
P2Y12 receptor human	Polyclonal	324–342	Microglia	1:200	Anaspec

[a]*Not specified in the data sheet.*

antibodies (**Table 3**) in 0.3% Triton X-100 and 2% NDS in PBS, for 24–48 h at 4°C and processed for double and triple immunofluorescence. The secondary antibodies in 0.3% Triton X-100 and 2% NDS in PBS were Cy3-conjugated donkey anti-goat IgG (1:100, Jackson Immunoresearch, West Grove, PA, USA, red), Cy5-conjugated donkey anti-mouse IgG (1:100, Jackson Immunoresearch, blue), Alexa Fluor® 488-AffiniPure donkey anti-mouse IgG (1:200, Jackson Immunoresearch, green), and Alexa Fluor® 488-AffiniPure donkey anti-rabbit IgG (1:200, Jackson Immunoresearch, green). In the case of biotinylated primary antibody CD14 and Lectin from *Lycopersicon esculentum* (tomato) biotin conjugate (1:200, Sigma-Aldrich), Cy2-streptavidin conjugated secondary antibodies (1:200, Invitrogen) were used.

Rat monocytes maintained in culture for 1 week were fixed with 4% paraformaldehyde for 20 min, permeabilized with 0.1% Triton X-100 for 10 min, blocked in PBS/1% bovine serum albumin, and incubated in PBS/1% bovine serum albumin with anti-cluster of differentiation 68 (CD68, 1:200, Santa Cruz Biotechnology, CA, USA). The secondary antibody was Cy3-conjugated donkey anti-rabbit IgG (1:200, Jackson Immunoresearch). Cells were stained with Höechst 33342 (1:1,000) and covered with coverslip in Fluoromount medium (Sigma-Aldrich) for confocal microscopy.

Quantification of CD68 immunoreactivity was performed with monocytes from rat spleen (from an average of six fields for each animal in each group), using Image J software. Data are expressed as optical density with respect to CFA group.

Confocal Microscopy

Immunofluorescence analysis was performed by confocal laser scanning microscope (Zeiss, LSM700; Iena, Germany) equipped with four laser lines: 405, 488, 561, and 639 nm. Brightness and contrast were adjusted with Zen software (Zeiss).

Protein Extraction and Western Blotting

Six different snap-frozen blocks of frontal cortex from three independent SPMS cases, and one block from five different control cases were processed for protein extraction. Detergent-soluble proteins were extracted with Ripa buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS in PBS, containing protease inhibitors), using a micropestle. After a short sonication, the homogenates were incubated on ice for 1 h and centrifuged at 13,000 rpm for 10 min at 4°C. To extract detergent-insoluble proteins, the resulting pellet was resuspended in 0.5 M Tris HCl (pH 6.8) containing 2% w/v SDS (same volume as utilized for soluble protein extraction), and incubated at RT for 10 min. Insolubilized material in the pellet

(15,000 rpm for 15 min) was discarded. Protein quantification was performed from the supernatants by Bradford colorimetric assay (Biorad, Milan, Italy). Proteins (15 μg) were separated by electrophoresis on 10% SDS-PAGE and transferred to nitrocellulose Hybond-C-extra membranes (Amersham Biosciences, Cologno Monzese, Italy). The filters were pre-wetted in 5% blocking agent in TBS-T (10 mM Tris pH 8, 150 mM NaCl, 0.1% Tween 20) and hybridized overnight with P2X7-extracellular epitope antibody (1:500, peptide KKGWMDPQSKGIQTGRC, corresponding to amino acids 136–152 of mouse P2X7 receptor, Alomone Labs), in the absence or presence of the neutralizing immunogenic peptide used in a 1:1 protein ratio, and with monocyte chemoattractant protein-1 (MCP-1) antibody (1:1,000, ThermoFisher Scientific). The signals were detected with anti-rabbit horseradish peroxidase-conjugated antibody (1: 5,000), and developed by enhanced chemiluminescence (Amersham Biosciences), using Kodak Image Station (KDS IS440CF) and semi-quantitative analysis of which was performed with Image J software.

Total proteins from control ($n = 4$), CFA ($n = 3$), and EAE ($n = 4$) cultured rat monocytes and from control C57BL/6 cultured mouse monocytes ($n = 3$) were obtained after lysis of cells on ice for 1 h in Ripa Buffer and centrifugation at 14,000 rpm for 10 min at 4°C. Protein quantification was performed from the supernatants by Pierce™ BCA Protein Assay Kit (Thermo Scientific, Monza, MB, Italy). Proteins were separated by electrophoresis on 10% SDS-PAGE, transferred to nitrocellulose membranes, and processed for western blotting using P2X7-intracellular epitope antibody [1:500, peptide (C) KIRKEFPKTQGQYSGFKYPY, corresponding to amino acids 576–595 of rat P2X7 receptor, Alomone Labs]. The signal was detected with anti-rabbit horseradish peroxidase-conjugated antibody (1: 2,500), and developed by enhanced chemiluminescence (Amersham Biosciences). The bands of interest were visualized using Kodak Image Station (KDS IS440CF) and semi-quantitative analysis was performed with Image J software.

Statistical Analysis

Data are presented as mean ± SEM and statistical analysis was determined by ANOVA. Statistical differences between groups were verified by Student's *t*-test. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{****}p < 0.0001$ were considered statistically significant.

RESULTS

P2X7 Receptor Is Down-Regulated in Monocytes during Acute MS and EAE

Given the abundant expression of P2X7R on monocytes (38–40), in this work we firstly confirmed its presence in circulating monocytes from HD and then demonstrated its down-regulation in stable and acute MS patients compared with HD, by RT-qPCR (**Figure 1A**). Furthermore, FACS analysis confirmed a significant decrease of CD14/P2X7R-positive monocytes only in MS acute patients compared with stable MS and HD conditions (**Figure 1B**).

Because the rat model of EAE is one among the most commonly used animal model for studying MS pathogenesis, by resembling particularly the acute form of the disease (41), we observed also in EAE monocytes, compared with CFA, a statistically significant decrease of P2X7R mRNA by RT-qPCR (Figure S1A in Supplementary Material), and of protein content by western blot analysis (Figure S1B in Supplementary Material). This occurs in parallel to the increase of the specific monocyte inflammatory marker IL-6 mRNA (Figure S1C in Supplementary Material), and of CD68 protein (Figure S1D in Supplementary Material).

Inflammatory Stimuli Down-Regulate P2X7 Receptor in Purified Human, Rat, and Mouse Monocytes

In order to mimic an inflammatory insult as it occurs in MS, monocytes from HD were challenged *ex vivo* with LPS or BzATP for 4 and 24 h. A significant decrease of human P2X7R/CD14 bearing monocytes occurred after treatment for 24 h with LPS (**Figures 1C,E**, ~48% reduction) or BzATP (**Figures 1D,E**, ~63% reduction), as shown by FACS analysis. Western blot analysis confirmed these results *in vitro* in rat cultured monocytes, showing down-regulation of P2X7R protein after 4 and 24 h of BzATP (Figure S1E in Supplementary Material, ~60 and 90% reduction, respectively) or LPS stimulation for 24 h (Figure S1E in Supplementary Material, ~45% reduction). Similar results were also obtained with mouse purified monocytes challenged *in vitro* with LPS or with BzATP. (Figure S1F in Supplementary Material).

P2X7 Receptor Is Present on Monocytes in Blood Vessels of SPMS Frontal Cortex

By immunohistochemistry and immunofluorescence analysis, we next analyzed the cortical tissue from 13 different cases of SPMS for the presence of P2X7R immunoreactivity and colocalization with specific cellular markers (**Tables 2** and **3**). In detail, we examined 1–2 different tissue blocks from all cases and inspected 4–10 different slices for each block. The tissue slices were studied in areas either presenting neuronal injury/inflammation, or devoid of visible damage. We observed typical features of cortical demyelination and inflammation in all SPMS cases analyzed. Independent analysis was also performed in cortical tissue from four patients who died by non-neurological diseases (data not shown). In particular, by immunohistochemistry (**Figure 2A**) the P2X7R immune-positive signal was found to delineate the plasma membrane of roundish cells distributed in distinct clusters in the cortical parenchyma. By immunofluorescence analysis, this roundish P2X7R immune-positive signal (red) was found to colocalize with the CD45 leukocyte marker (**Figure 2B**, green). By staining with Lectin (green) that specifically delineates endothelial vascular cells, we thus concluded that the roundish P2X7R-positive cluster cells were likely located inside blood vessels (**Figures 2C,D**) within the SPMS cortical parenchyma. Similar results were confirmed in all cases analyzed (**Table 2**) and in donors not deceased by neurological conditions (data not shown). By performing double

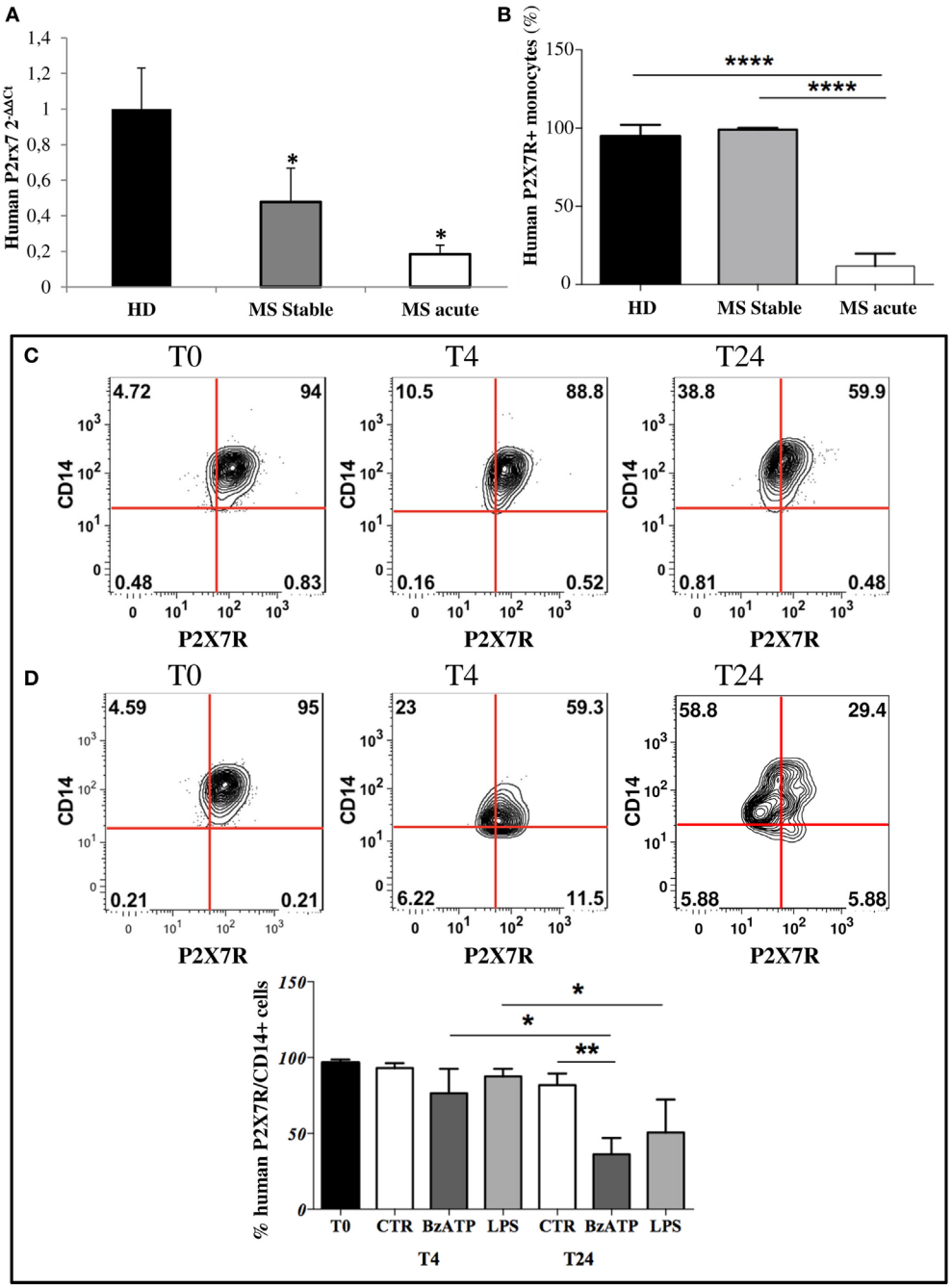

FIGURE 1 | P2X7 receptor (P2X7R) is down-regulated on circulating multiple sclerosis (MS) monocytes and on healthy donors (HD) monocytes after *in vitro* induced inflammation. **(A)** RT-qPCR analysis of P2X7R was performed with freshly isolated monocytes from MS stable ($n = 8$), acute patients ($n = 8$), and HD ($n = 8$). GAPDH was used for normalization. **(B)** Flow cytometry analysis was used to isolate cluster of differentiation 14 (CD14)-positive monocytes and P2X7R-CD14 double-positive cells within freshly isolated peripheral blood mononuclear cells from acute, stable MS patients, and HD. Cumulative data of P2X7R-positive cells within monocytes are reported as % mean ± SEM ($n = 5$). Statistical significance was calculated by ANOVA-Student's *t*-test, $^{****}p < 0.0001$ and $^{*}p < 0.05$. Circulating monocytes purified from HD were incubated with inflammatory lipopolysaccharide (250 ng/ml) **(C)** or 2′3′-*O*-(4-benzoyl-benzoyl)adenosine5′-triphosphate (250 μM) **(D)** for 4 and 24 h. Flow cytometry analysis and representative plots of P2X7R expression are shown **(C,D)**, together with cumulative data from three independent experiments are presented **(E)**. Statistical significance was calculated by ANOVA-Student's *t*-test $^{*}p < 0.05$, $^{**}p < 0.01$.

and triple confocal immunofluorescence of these cluster cells with the CD14 monocyte/macrophage marker (**Figures 2E–G**, green), we demonstrated only partial colocalization with P2X7R immunoreactivity (**Figures 2E–G**, in red), being the P2X7R signal apparently absent from CD14-positive cells (white arrows) that are proximal to the endothelium of the blood vessels and

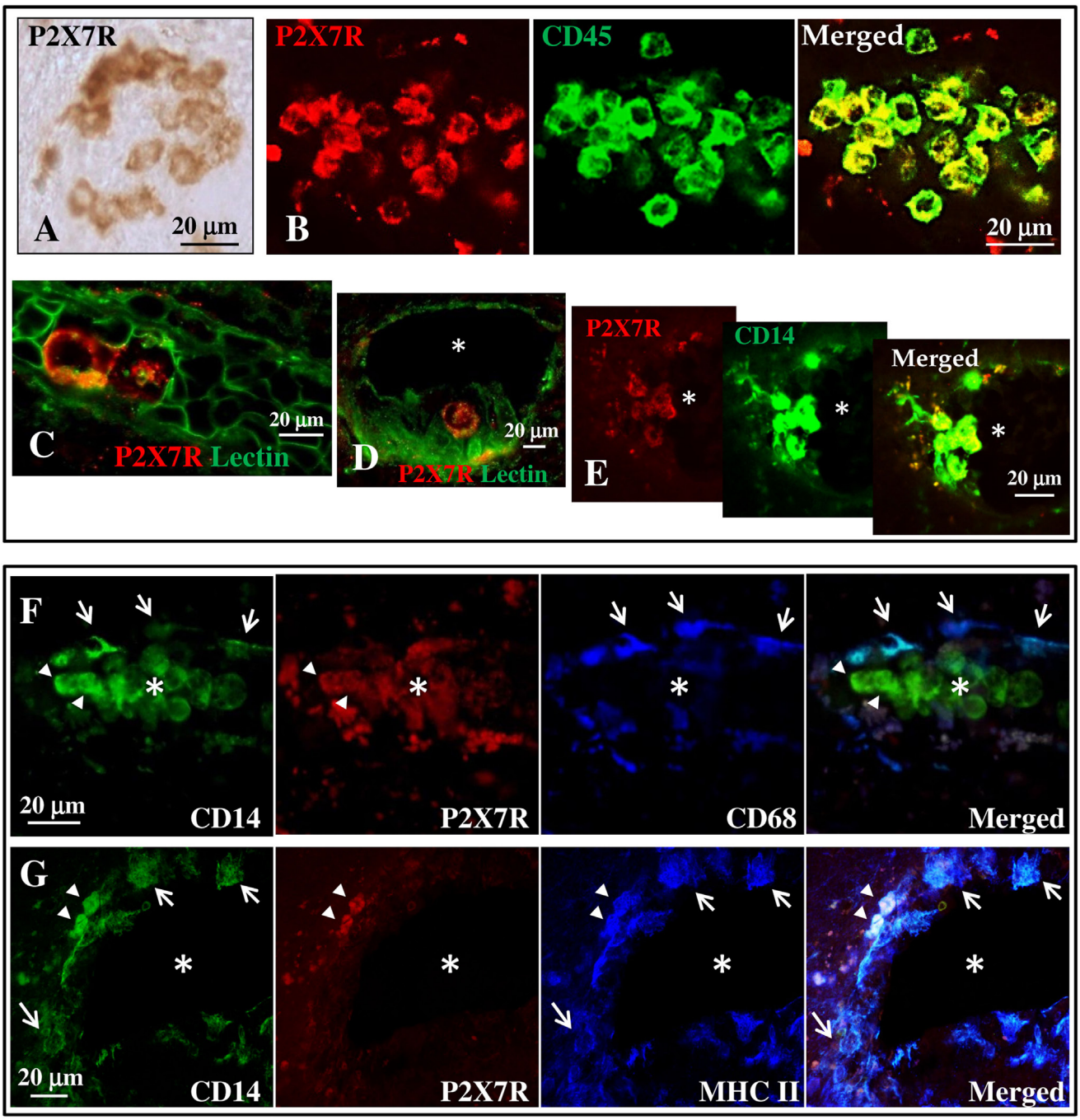

FIGURE 2 | P2X7 receptor (P2X7R) is present on monocytes in blood vessels of secondary progressive multiple sclerosis (SPMS) frontal cortex. **(A)** Immunohistochemistry on sections from human frontal cortex shows roundish P2X7R-positive cells distributed within distinct clusters throughout the entire SPMS tissue. Confocal double immunofluorescence analysis of these clusters exhibits colocalization of P2X7R protein (red) with CD45 leukocyte marker [**(B)**, green]. Staining with Lectin from *Lycopersicon esculentum* for vascular endothelial cells [**(C,D)**, green] clearly shows the presence of P2X7R-positive roundish cells (red) within the lumen of blood vessels (asterisk). Double immunofluorescence of P2X7R-positive clusters shows colocalization of P2X7R (red) with cluster of differentiation 14 (CD14) monocyte/macrophage marker [**(E)**, green]. Confocal triple immunofluorescence analysis is then performed with antibodies for CD14 [**(F,G)**, green], P2X7R [**(F,G)**, red], and microglia/macrophages markers CD68 **(F)** or major histocompatibility complex II [**(G)**, blue]. The asterisk always indicates the lumen of blood vessels, arrows indicate P2X7R-negative cells, and arrowheads P2X7R-positive cells.

that are simultaneously positive for CD68 (**Figure 2F**, blue, white arrows) or for the major histocompatibility complex II (MHC II) (**Figure 2G**, blue, white arrows) macrophage/microglia markers. In all the sections analyzed, P2X7R is present only on few perivascular double CD14- and MHC II-positive cells (**Figure 2G**, arrowheads, white signal).

P2X7 Receptor Is Present on Astrocytes in the Parenchyma of SPMS Frontal Cortex

We next investigated the distribution of P2X7R in the cortical parenchyma outside from the blood vessels. Double immunofluorescence confocal analysis indicated the absence of colocalization of P2X7R (red) with P2Y12R (green) or MHC II (blue) (markers, respectively, of quiescent or reactive/active macrophages/microglia, **Figures 3A,B**) (42, 43), or with myelin

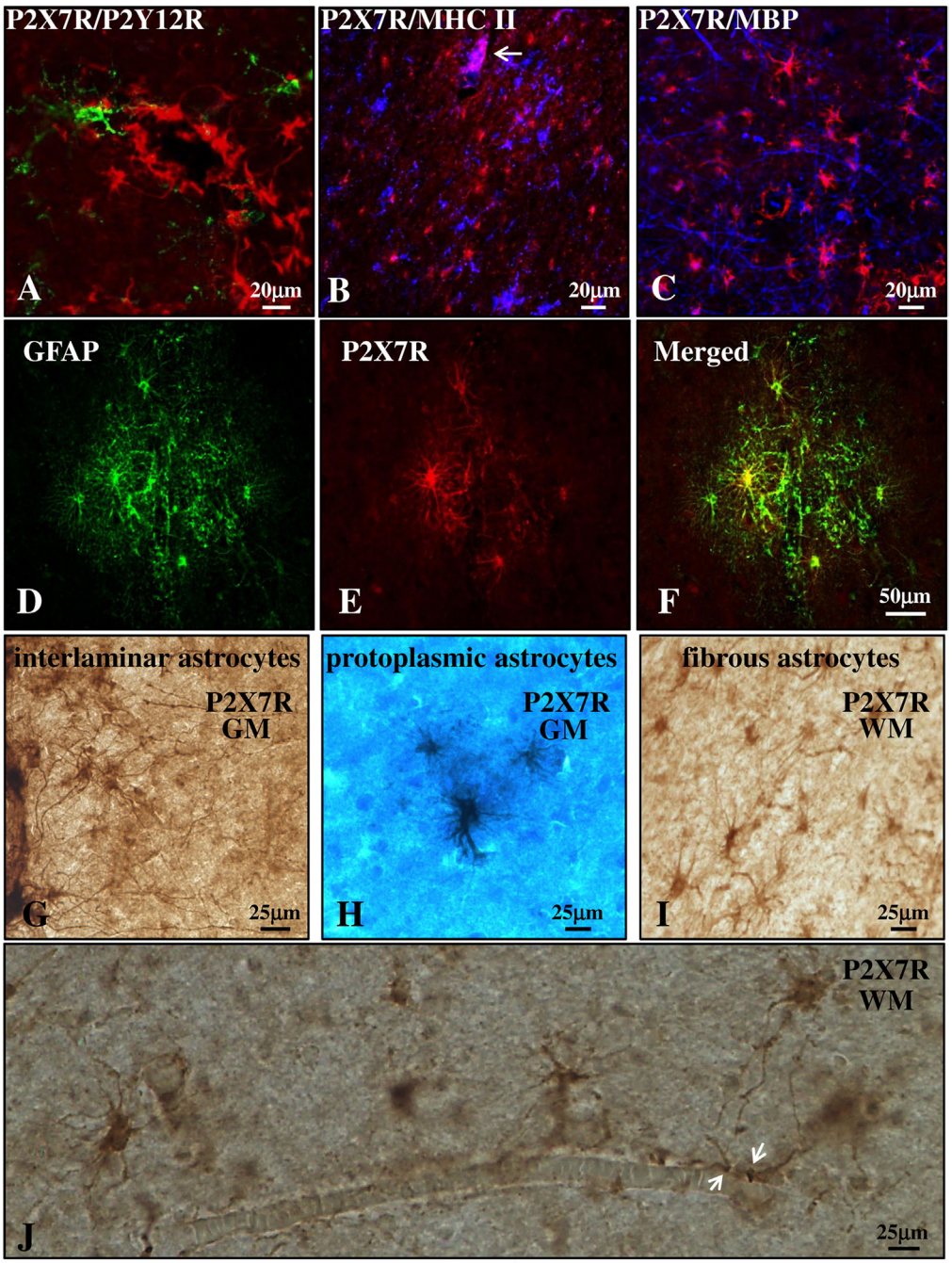

FIGURE 3 | P2X7 receptor (P2X7R) is present on astrocytes in the parenchyma of secondary progressive multiple sclerosis (SPMS) frontal cortex. Confocal analysis of SPMS frontal cortex parenchyma shows absence of colocalization of P2X7R (red) with P2Y12R [**(A)**, green], major histocompatibility complex II (MHC II) [**(B)**, blue], and myelin basic protein [**(C)**, blue], but the presence of merged P2X7R/glial fibrillary acidic protein signals **(D–F)**. P2X7R/MHC II-positive signal is also visible but confined in the lumen of a blood vessel [**(B)**, arrow, pink]. Immunohistochemistry analysis of cortical parenchyma reveals the abundant presence of P2X7R in GM on interlaminar **(G)** and protoplasmic astrocytes **(E)**, and in white matter on fibrous astrocytes **(I,J)**. In **(J)**, astrocytic "vascular feet" are visible adjacent to the blood vessel walls (arrows).

basic protein (MBP) (blue, marker of myelin fibers, and oligodendrocyte cell bodies, **Figure 3C**). The receptor was instead strongly expressed in glial fibrillary acidic protein (GFAP)-positive astrocytes present in both gray (GM, **Figures 3D–F**) and white matter (WM) (not shown) of both control and MS patients. Moreover, immunohistochemistry studies corroborated the presence of P2X7R in the soma and fibers of interlaminar astrocytes (**Figure 3G**) present in GM cortical layer I that spread prominent, long, and unbranched processes throughout the layers of the cortex, and moreover in protoplasmic astrocytes (**Figure 3H**) that are well-organized in GM into non-overlapping spatial domains. In WM, we also observed a strong P2X7R signal in fibrous astrocytes (**Figure 3I**) that exhibit unbranched cellular processes and that often protrude "vascular feet" (44) that are physically connected to the external capillary walls (**Figure 3J**).

With the aim of further characterizing P2X7R expression and modulation in astrocytes, we acquired images from adjacent immunohistochemical fields within the same cortical sections and compared them with control cases (**Figures 4A–C**). Respect to control (**Figure 4A**), we observed a remarkable increase in P2X7R-positive astrocytes in different WM zones of SPMS cortical tissue presenting high levels of astrogliosis (**Figure 4B**) and glial scar formation (**Figure 4C**). In detail, in SPMS cortex, P2X7R immunoreactivity distinguished an area (**Figure 4B**) with strong reactive astrogliosis, intense cellularity, prominent hypertrophy, proliferation, and overlapping of astrocyte processes causing the disruption of distinctive astrocyte domains. Furthermore, the P2X7R signal also identified a zone very rich in parallel and interconnected fibers highlighting a prominent glial scar, where astrocytes displayed densely intersecting processes characterized by intense double P2X7R/GFAP-positive immunoreactive signal (**Figure 4C**; Figure S2 in Supplementary Material).

Expression of P2X7 Receptor in Active or Inactive Subpial Lesions of SPMS Frontal Cortex

Although MS is still widely considered a WM disease, demyelination is also prominent in GM. SPMS phase is characterized by subpial lesions that are highly abundant in progressive stages of MS, closely to the subarachnoid space, involving either part of a cortical gyrus, or often covering adjacent gyri (45). By triple immunofluorescence confocal analysis, we have characterized a subpial lesion with various degrees of inflammatory reaction and demyelination and observed different features of P2X7-positive immunoreactivity. In detail, the presence of profuse reactive MHC II-positive monocytes/macrophages/microglia (blue, **Figure 5B**) and the permanence of MBP-positive myelin fibers (green, **Figure 5C**) defined a chronic active lesion characterized by abundant P2X7R-positive signal (red, **Figure 5A** and insets) that highlighted a zone of intense cellularity and astrogliosis (see merged P2X7R-GFAP signal in the inset). On the other hand, in an area where MHC II (blue, **Figure 5E**) and MBP (green, **Figure 5F**) signals both decreased indicating a chronic inactive lesion (46), the P2X7R-positive immunoreactivity (red, **Figure 5D**) identified a zone very rich in fibers typical of a glial scar (see merged P2X7R-GFAP signal in the inset).

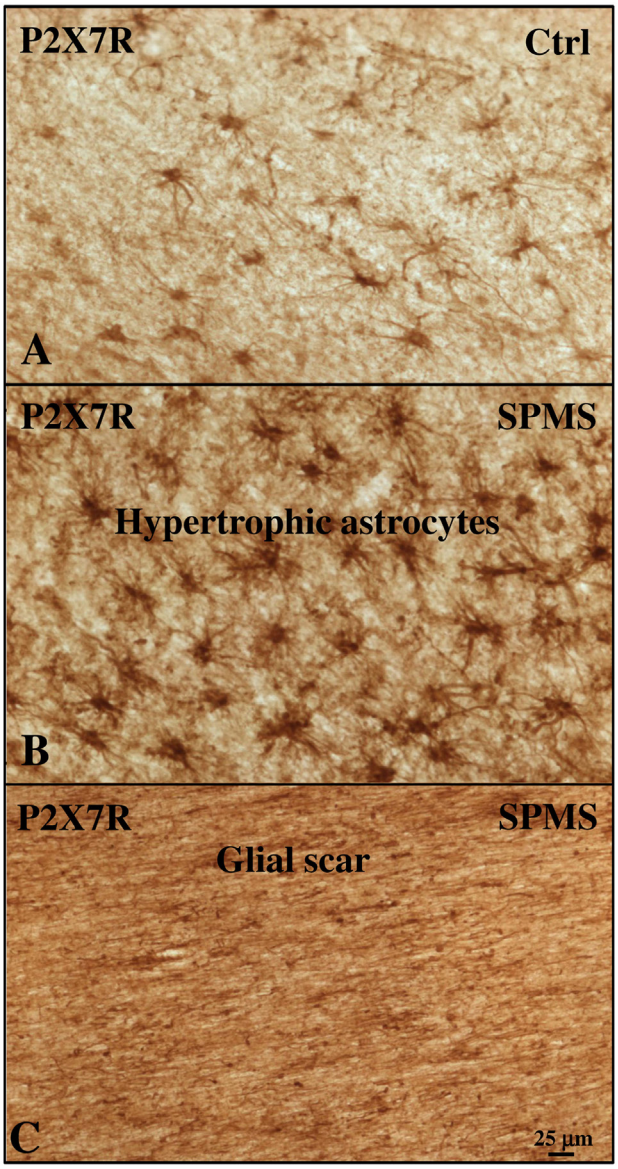

FIGURE 4 | P2X7 receptor (P2X7R) immunoreactivity is increased with astrogliosis in white matter (WM) of secondary progressive multiple sclerosis (SPMS) frontal cortex parenchyma. Immunohistochemistry analysis of two adjacent WM fields from SPMS frontal cortex characterized, respectively, by hypertrophic astrocytes **(B)** and glial scar **(C)** reveals a noteworthy increase in P2X7R-positive astrocytes **(B,C)**, compared with control case **(A)**.

MS Pathology Alters P2X7 Receptor mRNA and Protein Levels

To further investigate if SPMS progression modifies the P2X7R content, we analyzed total cell extracts from frontal cortex, by RT-qPCR and western blotting. A statistically significant increase of P2X7R mRNA was observed in SPMS patients, compared with controls (**Figure 6A**). Similar results were confirmed by immunoblotting with a P2X7R antibody raised against an extracellular epitope of the mouse P2X7R (corresponding to amino acid 136–152). In human frontal cortex tissue, we recognized

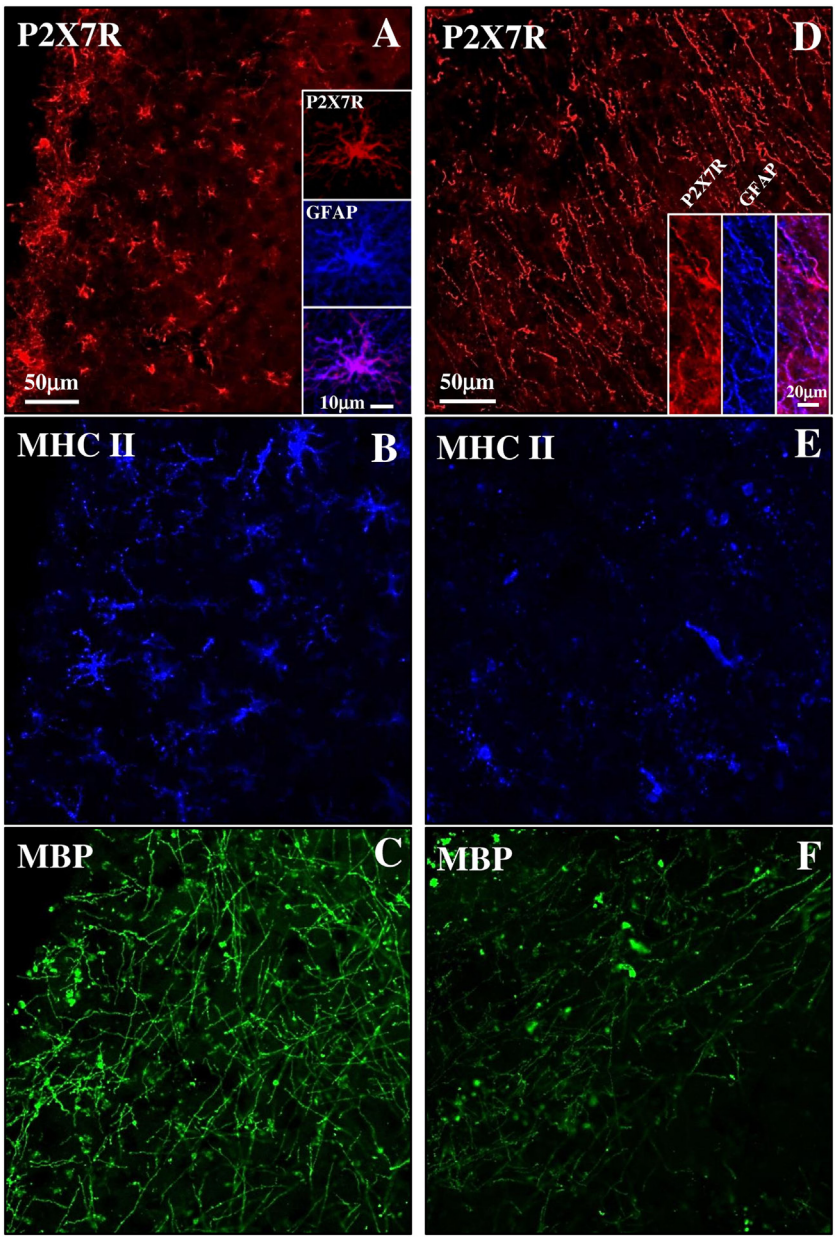

FIGURE 5 | P2X7 receptor (P2X7R) expression in both active and inactive subpial lesions of secondary progressive multiple sclerosis (SPMS) frontal cortex. Confocal triple immunofluorescence analysis performed with antibodies for P2X7R [**(A,D)**, red], major histocompatibility complex II (MHC II) [**(B,E)**, blue], and myelin basic protein (MBP) [**(C,F)**, green] on SPMS frontal cortex sections, shows a chronic active subpial lesion **(A–C)** with abundant glial fibrillary acidic protein (GFAP)/P2X7R-positive signal **(A**, inset**)**, with reactive MHC II-positive monocytes/macrophages/microglia (blue) and with MBP-positive myelin fibers (green). In a chronic inactive lesion **(D–F)**, an intense GFAP/P2X7R glial scar is shown [**(D)**, inset], with decreased MHC II-positive [**(E)**, blue] and MBP-positive [**(F)**, green] immunoreactivities.

three specific protein bands with estimated sizes in the ranges 52–72, 72–95, and 95–140 kDa, perhaps corresponding to the different P2X7 isoforms, and that were moreover abolished by the P2X7R neutralizing immunogenic peptide (data not shown). In addition, particularly the 95–140 kDa P2X7R was found significantly up-regulated in both detergent-soluble and -insoluble fractions of SPMS tissue extracts respect to controls, while the 52–72 kDa P2X7R was significantly up-regulated only in the detergent-insoluble fraction of SPMS extracts, with a trend to increase in the detergent-soluble fraction (**Figures 6B,C**).

P2X7 Receptor Colocalizes with MCP-1 Chemokine in Human Frontal Cortex

Because the up-regulation of MCP-1 in astrocytes is demonstrated to have an important role in recruiting leukocytes in the

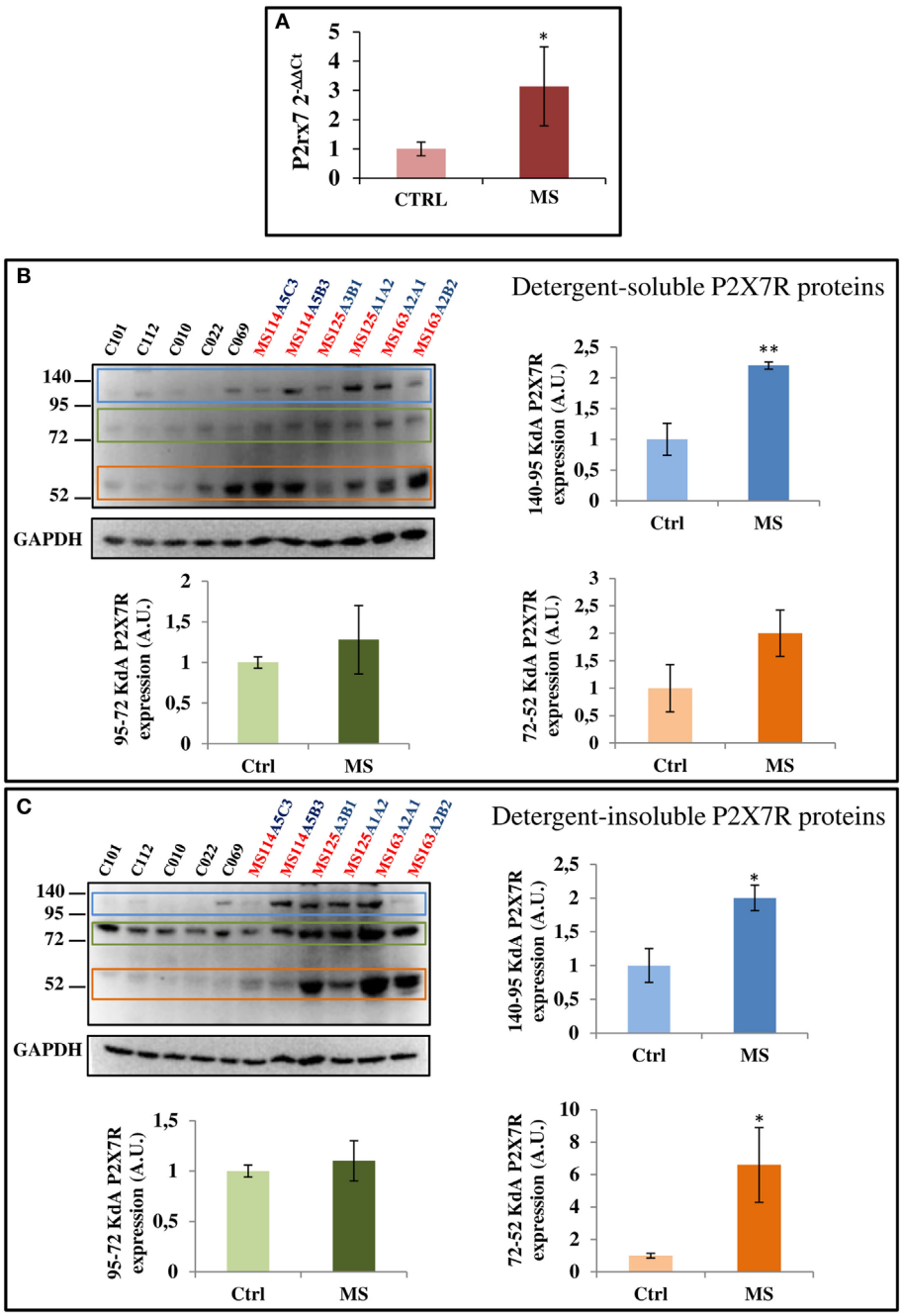

FIGURE 6 | P2X7 receptor (P2X7R) mRNA and protein are augmented in secondary progressive multiple sclerosis. **(A)** Total RNA was extracted from three different snap-frozen blocks from MS patients (cases MS114, MS125, and MS163) and six controls (cases C12–101, C12–112, C13–010, C13–022, C14–069, and C14–053) and the expression of P2X7R mRNA was examined by RT-qPCR. Cortical protein extracts (15 μg/well) from different tissue blocks (A5C3, A5B3, A3B1, A1A2, A2A1, and A2B2) of MS cases MS114, MS125, and MS163 were analyzed by western blotting for the modulation of P2X7R, with respect to control cases (C12–101, C12–112, C13–010, C13–022, and C14–069), in both detergent-soluble **(B)** and -insoluble fractions **(C)**. GAPDH was used for protein normalization. Statistical significance was calculated by Student's *t*-test, $^{*}p < 0.05$, $^{**}p < 0.01$. Results are shown as mean ± SEM.

CNS during MS (47–49), and the P2X7R agonist BzATP increases MCP-1 expression in astrocytes through P2X7R activation (50), we evaluated the expression of this chemokine in our human SPMS cortical tissue and its potential colocalization with P2X7R on astrocytes. Western blot analysis of protein extracts from control and SPMS patients, demonstrated a strong up-regulation (about twofold increase) of MCP-1 in MS respect to control (**Figure 7**). Moreover, triple immunofluorescence confocal analysis showed an unambiguous intense colocalization (white signal) among P2X7R (red), MCP-1 (green), and GFAP (blue)

proteins, both in control (**Figures 8A–D**) and SPMS tissues (**Figures 8E–H**), and moreover a strong up-regulation of both P2X7R and MCP-1 during astrogliosis occurring in WM of SPMS patients (**Figures 8E–H**).

DISCUSSION

The adhesion of leukocytes to endothelial cells and their migration into the CNS parenchyma through the blood–brain barrier is a critical step in the development of brain inflammation. Although many types of immune cells are involved in this process during MS progression, activated monocytes are believed to be one of the first phenotypes to reach the brain and initiate neuroinflammation. Because P2X7R is highly expressed in immune cells of the monocyte–macrophage lineage (38), and activation of P2X7R triggers multiple responses affecting the intensity and duration of innate immune and inflammatory reactions in lymphoid leukocytes (51, 52), the aim of this work was to characterize the P2X7R in MS peripheral monocytes and cortical parenchyma. We found that P2X7R protein expression is down-regulated during the acute phase of the disease both in patients and rat EAE monocytes and, moreover, the protein levels of the receptor are reduced in human, rat, and mouse healthy monocytes challenged *in vitro* with pro-inflammatory stimuli. Based on these results, we can hypothesize that high-P2X7R expression might be perhaps deleterious for monocyte survival, and therefore the receptor might be reduced during MS to contribute to initiate and propagate the neuroinflammation. Confirming our hypothesis, overexpression of P2X7R is induced in human monocytes/macrophages infected with high-apoptogenic *M. tuberculosis*, in a suicide-leading track as an attempt to reduce mycobacterial viability (53). Similarly, P2X7R activation with resultant Ca^{2+} overload triggers death also of native mouse monocytes/macrophages (18). Finally, Treg cells with high levels of P2X7R expression are prompted to die (54) when the clearance of excessive toxic ATP is less efficient, as in MS patients with reduced levels of the CD39 ectonucleotidase enzyme (55). Therefore, when high-extracellular concentrations of ATP are released in damaged areas as a result of tissue injury, the down-regulation of P2X7R expression as we demonstrated here, seems an attempt to limit a long-lasting opening of P2X7R channel and massive Ca^{2+} entry, with the final aim of sustaining monocyte survival and, in the case of MS, enhancing pro-inflammatory signals and further damage into the CNS. While this mechanism would apparently diverge from what observed in Behçet's disease where P2X7R is instead up-regulated in monocytes from patients (56), it is however conserved in pathological conditions other than MS, and in additional cell phenotypes. Reduction of P2X7R expression in PBMCs leading to intracellular calcium dysregulation occurs for instance during ALS (40), a neuroinflammatory/neurodegenerative disease also involving P2X7R (14, 57, 58); $P2X7R^{-/-}$ oligodendrocytes show increased survival in EAE (31), and increased survival of oligodendrocyte precursors occurs also after down-regulation of P2X7R during hypoxia ischemia (59).

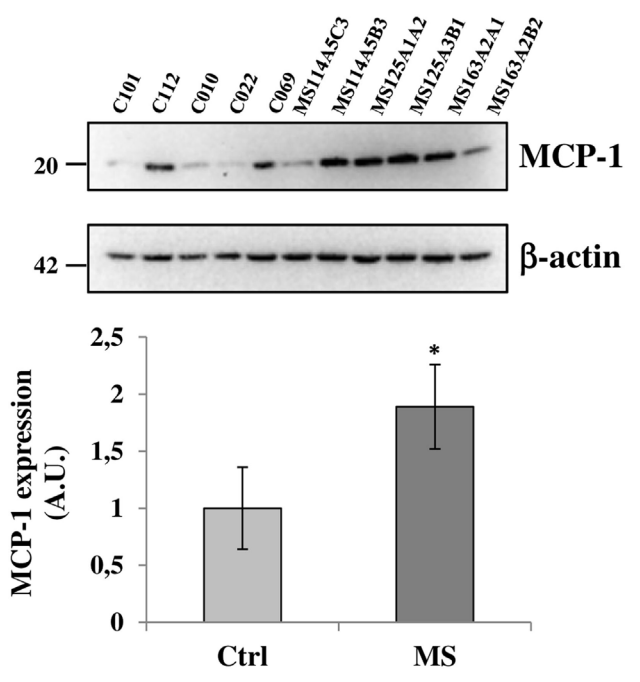

FIGURE 7 | Monocyte chemoattractant protein-1 (MCP-1) chemokine is up-regulated in secondary progressive multiple sclerosis frontal cortex. Equal amount of total protein extracts of cortical tissue (15 μg/well) from different tissue blocks (A5C3, A5B3, A3B1, A1A2, A2A1, and A2B2) of multiple sclerosis (MS) cases MS114, MS125, and MS163 was analyzed by western blotting for the expression of MCP-1 chemokine with respect to control cases (C12–101, C12–112, C13–010, C13–022, and C14–069). β-actin was used for protein normalization. Statistical significance was calculated by Student's *t*-test, $^*p < 0.05$. Results are shown as mean ± SEM.

We have next looked at P2X7R expressed in CNS tissue, and found that the P2X7R is localized on CD45/CD14-positive monocytes that are visible in the lumen of blood vessels of the cortical parenchyma. Remarkably, the receptor was progressively lost on both CD14/CD68- or CD14/MHC-II-positive cells neighboring the endothelium of the blood vessels and perhaps entering into the CNS, thus corroborating the hypothesis that inflammatory stimuli in the peripheral tissue might trigger a secondary autocrine/paracrine down-regulation of P2X7R expression, with the final aim of boosting and propagating the neuroinflammation into the CNS.

Although these results validate the renowned importance of purinergic P2X7R-dependent signaling in neuroinflammatory conditions, the impact of this receptor in the pathogenesis and clinical aspects of MS is still to be defined. In order to clarify how P2X7R down-regulation in monocytes might correlate to inflammatory lesions and disease progression, we investigated its expression also in autoptic cortical tissue from SPMS donors. In contrast to optic nerve from rat EAE (31) and MS patients (32), in SPMS frontal cortex we found P2X7R absent from myelin fibers and oligodendrocyte cell bodies. Differently from MS spinal cord (21), in SPMS frontal cortex we did not detect P2X7R expression on resting and activated microglia. In contrast to other MS cerebral areas, in SPMS frontal cortex we found P2X7R also absent from neurons, although we confirmed its presence in astrocytes (23). In particular, abundant P2X7R immunoreactivity was found localized on interlaminar

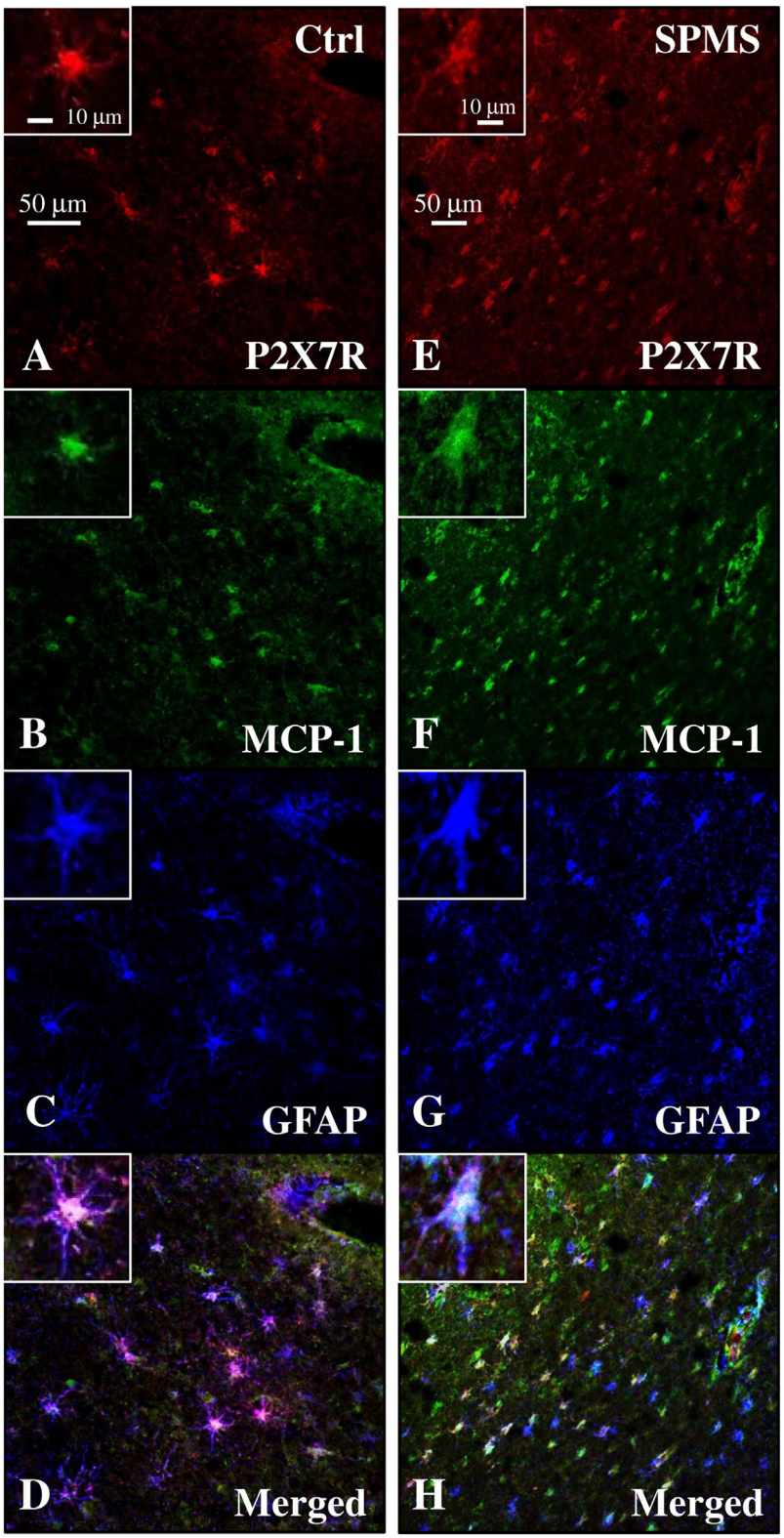

FIGURE 8 | Monocyte chemoattractant protein-1 (MCP-1) chemokine colocalizes with P2X7 receptor (P2X7R) on astrocytes in secondary progressive multiple sclerosis (SPMS) frontal cortex. Triple immunofluorescence confocal analysis performed on sections of frontal cortex from control **(A–D)** and SPMS **(E–H)** with antibodies for P2X7R [**(A,E)**, red], MCP-1 [**(B,F)**, green], and glial fibrillary acidic protein [**(C,G)**, blue] shows colocalization [**(D,H)**, white signal] and strong up-regulation of signals in white matter of SPMS **(E–H)**.

and protoplasmic astrocytes of gray matter, and on fibrous astrocytes of white matter. Noticeably, P2X7R-positive astrocytes augmented in cortical tissue of SPMS patients in proximity of lesions. By further extending previous results, these observations thus indicate that P2X7R localization in the CNS and modulation in MS is strictly tissue- and specie-specific.

Being P2X7R *per se* involved in several and sometimes opposite functions (15), its presence on astrocytes playing a dual role in MS by either promoting inflammation and impeding tissue repair, or protecting healthy tissue from adjacent zones of strong inflammation (60), can designate its potential role in several distinct actions. Indeed, we might hypothesize that in astrocytes of SPMS frontal cortex, the up-regulation of P2X7R might contribute to build the parenchyma micro-architecture, being the receptor expressed by interlaminar, protoplasmic, and fibrous astrocytes (61–63). P2X7R could also regulate extracellular K+ homeostasis and participate to the removal of excess glutamate, by directly modulating K+ efflux (64, 65). Moreover, increased P2X7R might influence the connectivity of neuronal circuits, being the receptor known to be involved in the control of myelination (29), or provide metabolic support to neurons, by regulating the lipid metabolism pathway (66). Finally, P2X7R might participate to preserve the blood–brain barrier (60), since we demonstrated here its expression in astrocytes protruding their vascular feet on external capillary walls. However, the specific role of P2X7R up-regulation on SPMS astrocytes might be regulated by several features, including the specific disease stage, the presence of active or inactive lesions characterized, respectively, by P2X7R-positive astrogliosis or

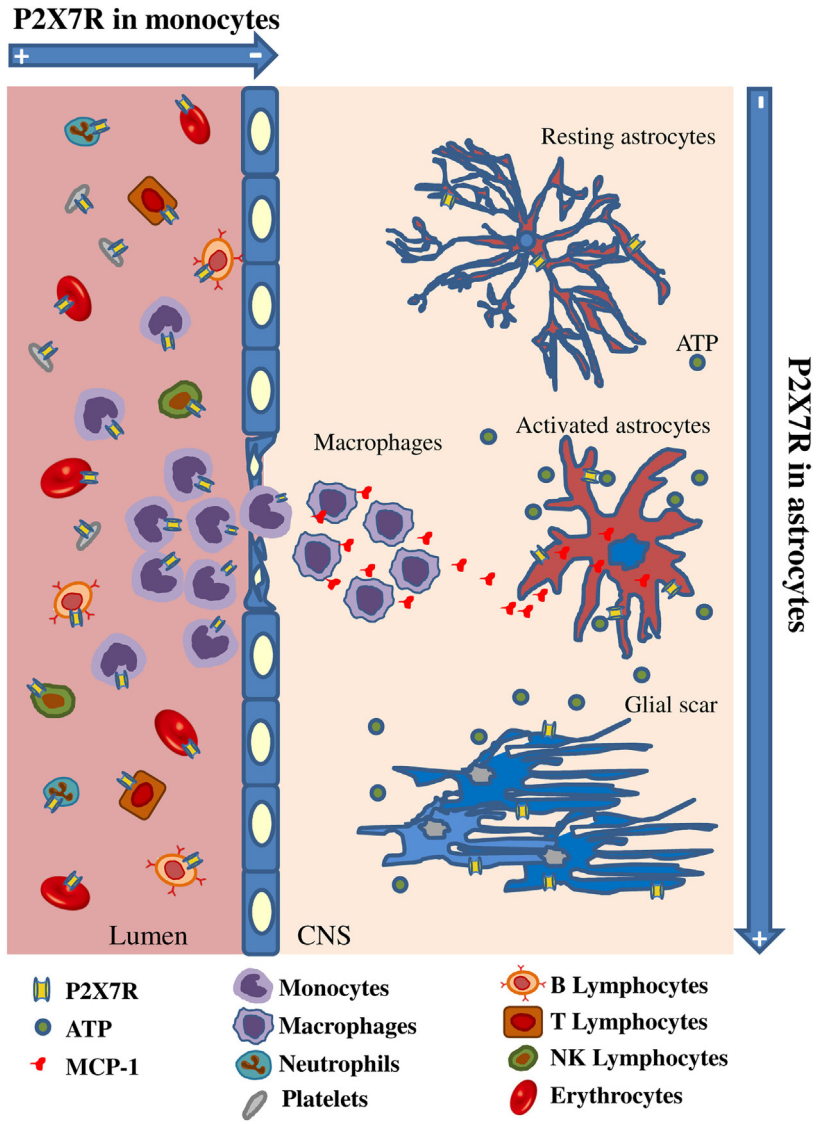

FIGURE 9 | Spatiotemporal profile of P2X7 receptor (P2X7R) expression in multiple sclerosis (MS). The cartoon describes that P2X7R is down-regulated in monocytes during their activation and extravasation from blood vessel to MS cerebral cortex, while the receptor and the monocyte chemoattractant protein-1 chemokine are up-regulated on MS astrocytes concurrently with increased glial fibrillary acidic protein signal and glial scar formation.

P2X7R-positive glial scar, the interaction with various cell types, such as for instance the endothelial cells. Extracellular soluble factors might also influence the diverse cellular reactivity to P2X7R activation during MS. For instance, extracellular ATP is known to induce the secretion from astrocytes of MCP-1 (50), a crucial chemokine up-regulated after CNS trauma (67), and known to have an important role in engaging monocyte-rich infiltrates into the CNS during MS (47–49). In addition, BzATP increases *in vitro* MCP-1 levels in cultured astrocytes directly through P2X7R activation (50). On this regard, the increase of MCP-1 protein that we have demonstrated in SPMS cortical extracts might likely be related to the up-regulation of P2X7R in astrocytes. Supporting this possibility, we have also demonstrated that MCP-1 is induced in cortical MS protein extracts and indeed colocalizes with P2X7R on astrocytes in SPMS frontal cortex.

By considering our results in the context of previous knowledge, we can therefore formulate the following hypothesis: extracellular ATP is increased in CNS tissue as an alarm signal due to progressive homeostasis loss during MS; astrocytes up-regulate P2X7R and MCP-1; this last functions as attractant for peripheral monocytes which in turn down-regulate P2X7R to guarantee their survival and invasion into the CNS tissue, thus contributing to the detrimental effects of neuroinflammation (**Figure 9**). Further work will verify our hypothesis and the time-cause correlation of these events. By gaining insights into P2X7R dynamics and signaling, we expect to contribute to further discern some molecular aspects of MS.

ETHICS STATEMENT

Blood samples were obtained following acquisition of the study participants' informed consent. The protocol was approved by ethic committees of San Camillo Forlanini Hospital. Patients enrolled were diagnosed with relapsing-remitting form of MS according to 2005-revised McDonald's criteria (34). Prefrontal cortex tissue was collected postmortem by UK MS Tissue Bank at Imperial College, London, and procedures for retrieval, processing, and storage have gained ethical approval. Animal procedures were performed according to European Guidelines for animal use in research (86/609/CEE) and requirements of Italian laws (D.L. 116/92), according to protocols no. 112/2014B and no. 119/2015PR by Italian Ministry of Health. Efforts were made to minimize animal suffering and the number of animals used.

AUTHOR CONTRIBUTIONS

SAm and CV: study concept and design. SAm, CP, PF, EP, SAp, CM, FLP, LB, and CV: data acquisition and analysis. SR and CG: collection and selection of blood samples and/or clinical data. SAm, CP, EP, and CV: manuscript and/or figures drafting. SL participated in interpreting results and critical reading of the manuscript. LB supervised flow cytometry analysis and contributed with immunology expertise. All authors edited and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/article/10.3389/fimmu.2017.01529/full#supplementary-material.

FIGURE S1 | P2X7R is downregulated on spleen monocytes from rat EAE, and on rat and mouse spleen monocytes after pro-inflammatory induction with LPS and BzATP. CFA and EAE purified rat monocytes were subjected to RT-qPCR **(A)** and western blot analysis **(B)** for evaluation of P2X7R expression. Data in **(A,B)** represent means ± SEM ($n = 4$ in EAE and $n = 3$ in CFA). RT-qPCR **(C)** and immunofluorescence analysis **(D)**, respectively for IL-6 mRNA and CD68 protein is shown. Data represent means ± SEM ($n = 3$ in EAE, and $n = 3$ in CFA). Statistical significance was calculated by Student's *t*-test, $^{*}p < 0.05$ compared to CFA. Western blot analysis of P2X7R expression after *in vitro* administration of pro-inflammatory BzATP (250 μM) or LPS (100 ng/ml) for 4 and 24 h to purified rat **(E)** and mouse **(F)** monocytes. Equal amount of total protein (3–6 μg/well) was analyzed in each sample and GAPDH was used for protein normalization. Results are shown as means ± SEM, $n = 4$ for rat and $n = 3$ for mouse. Statistical significance was calculated by Student's *t*-test, $^{*}p < 0.05$, $^{**}p < 0.01$ compared to T0.

FIGURE S2 | P2X7R/GFAP-positive astrocytes characterize a glial scar in WM chronic lesion of SPMS frontal cortex. Confocal double immunofluorescence analysis performed with antibodies for P2X7R (red) and GFAP (blue) on SPMS frontal cortex sections, shows a chronic lesion in WM characterized by the presence of a glial scar with abundant P2X7R/GFAP double-positive fibers.

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Gene Expression Profiling of Multiple Sclerosis Pathology Identifies Early Patterns of Demyelination Surrounding Chronic Active Lesions

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In multiple sclerosis (MS), activated microglia and infiltrating macrophages phagocytose myelin focally in (chronic) active lesions. These demyelinating sites expand in time, but at some point turn inactive into a sclerotic scar. To identify molecular mechanisms underlying lesion activity and halt, we analyzed genome-wide gene expression in rim and peri-lesional regions of chronic active and inactive MS lesions, as well as in control tissue. Gene clustering revealed patterns of gene expression specifically associated with MS and with the presumed, subsequent stages of lesion development. Next to genes involved in immune functions, we found regulation of novel genes in and around the rim of chronic active lesions, such as *NPY*, *KANK4, NCAN, TKTL1*, and *ANO4*. Of note, the presence of many foamy macrophages in active rims was accompanied by a congruent upregulation of genes related to lipid binding, such as *MSR1, CD68, CXCL16*, and *OLR1*, and lipid uptake, such as *CHIT1, GPNMB*, and *CCL18*. Except *CCL18*, these genes were already upregulated in regions around active MS lesions, showing that such lesions are indeed expanding. *In vitro* downregulation of the scavenger receptors *MSR1* and *CXCL16* reduced myelin uptake. In conclusion, this study provides the gene expression profile of different aspects of MS pathology and indicates that early demyelination, mediated by scavenger receptors, is already present in regions around active MS lesions. Genes involved in early demyelination events in regions surrounding chronic active MS lesions might be promising therapeutic targets to stop lesion expansion.

Keywords: multiple sclerosis, microarray, active lesions, microglia, demyelination, scavenger receptor, lipid uptake

INTRODUCTION

Multiple sclerosis (MS) is a neurological disease characterized by focal demyelinating lesions in the central nervous system, leading to a variety of symptoms, including problems with motor control, numbness or tingling sensation, cognitive problems, depression, and fatigue. Both genetic and environmental factors play a role in the onset and progression of MS [reviewed in Ref. (1, 2)].

Demyelination in MS is mediated by activated microglia and infiltrating macrophages, and in brains and the spinal cord of MS patients, both (chronic) active lesions and inactive scars are found. It is not clear why MS lesions are active demyelinating and which mechanisms contribute to the halt of lesion activity.

Depending on the level of demyelination and microglia/macrophage activation, MS lesions are characterized as active, chronic active, or inactive (3, 4). Active lesions contain lipid-laden microglia/macrophages throughout the lesions, while chronic active MS lesions have a demyelinated sclerotic core, surrounded by a rim of foamy microglia/macrophages. A recent magnetic resonance imaging study showed that chronic active lesions expand in time (5), and it is thought that at some point, active lesions turn into inactive sclerotic scars. Moreover, we found that chronic active lesion load correlates with fast progression of the disease, illustrating the clinical implications of lesion expansion (Luchetti et al., submitted).

Identification of gene expression in presumed, subsequent stages of MS lesions will increase insight into the molecular mechanisms related to lesion activity and halt. Gene expression profiling studies so far, on tissue blocks containing MS lesions from limited numbers (3–5) of MS patients per study, showed overall upregulation of pro-inflammatory pathways (6–10) and oxidative injury (11). One gene expression analysis of normal appearing white matter (NAWM) in MS demonstrated upregulation of genes associated with immunosuppression and protective mechanisms, but also pro-inflammatory mechanisms, suggesting a state of low-level inflammation and an unsteady balance (12, 13).

Previously, we analyzed differential gene expression between rims and regions surrounding chronic active and inactive MS lesions in substantial numbers of well-characterized MS brain donors by quantitative polymerase chain reaction (qPCR) and identified downregulation of macrophage inhibitory molecules around chronic active lesions (13). In this follow-up study, we set out a hypothesis-free microarray approach to study gene expression in rims and peri-rim regions of and around chronic active and inactive MS lesions from 15 MS patients and white matter (WM) of 10 matched control subjects. We identified gene expression specifically related to MS and to the assumed, subsequent stages of lesion development. Strikingly, genes connected with lipid binding and uptake were increased in the rim and peri-rim of chronic active lesions.

MATERIALS AND METHODS

Human Tissue

Post-mortem human brain tissue was provided by the Netherlands Brain Bank (NBB, Amsterdam, The Netherlands[1]). Informed consent was obtained from donors for brain autopsy and the use of tissue and clinical information for research purposes. At the time of death, 12 patients had relapsing-remitting course of the disease, 1 had a primary-progressive disease course, and for 2, the disease course could not be determined. MS diagnosis was confirmed post-mortem by a neuropathologist. One-way ANOVA analysis (Kruskal–Wallis test) showed no significant difference in age, post-mortem delay, or pH of cerebrospinal fluid (CSF) between the groups. Detailed donor characteristics are provided in **Table 1**; Table S1 in Supplementary Material.

Tissue Dissection and RNA Isolation

Cryostat sections were stained for myelin proteolipid protein (PLP; Serotec, Oxford, UK) and HLA-DP/Q/R (DAKO, Glostrup, Denmark) to assess MS lesion activity. Chronic active MS lesions were characterized by a sclerotic hypocellular demyelinated core, surrounded by a clear distinct rim of foamy HLA-positive macrophages (14). Inactive MS lesions were sclerotic demyelinated lesions without activated macrophages (3). Frozen chronic active and inactive MS lesions were cut in 20-µm sections using a cryostat and mounted on PALM MembraneSlides (PALM Microlaser Technologies, Munich, Germany). Every fifth to seventh section was stained with Sudan Black to confirm the lesion was still present and to facilitate dissection. Furthermore, every first and last section was stained for PLP and HLA-D/Q/R to assure continuous lesion activity.

The rim and peri-lesional (PL)-NAWM were dissected by laser dissection microscopy and stored in ice-cold TRIsure (Bioline, London, UK). Control tissue was dissected inside the cryostat using a pre-chilled scalpel and also stored in ice-cold TRIsure. After addition of chloroform and centrifugation, the aqueous phase was removed and mixed with an equal volume of 70% RNase-free ethanol. Samples were then applied to an RNeasy Mini column (Qiagen, Valencia, CA, USA) and further processed according to manufacturer's instructions. RNA yield

[1]http://www.brainbank.nl.

TABLE 1 | Donor characteristics per group.

Tissue type	Lesion area	Sex	Age	pH	PMD	RIN
Chronic active	Rim	2 M/5 F	49.4 ± 8.6	6.42 ± 0.17	8:24 ± 1:55	6.39 ± 0.67
	PL-NAWM					6.43 ± 0.33
Inactive	Rim	2 M/6 F	63.3 ± 11.7	6.43 ± 0.21	9:03 ± 0:45	5.79 ± 0.62
	PL-NAWM					6.16 ± 0.50
Control		3 M/7 F	59.7 ± 10.4	6.67 ± 0.35	8:23 ± 2:51	7.42 ± 0.67
One-way ANOVA			0.1351	0.3941	0.6958	0.0003

Age, age at death (years); F, female; M, male; pH, pH of CSF; PL-NAWM, peri-lesional normal appearing white matter; PMD, post-mortem delay (h:min); RIN, RNA integrity number. Data are represented as mean ± SD.

was determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), and quality was assessed on a Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA, USA). Only samples with RNA integrity number (RIN) values ≥5 were included. In total, 7 chronic active MS lesions, 8 inactive MS lesions, and WM of 10 control donors were included in this study. RIN values of control donors were significantly higher than of any of the MS lesion subareas. However, there was no difference in RIN value between the rim of chronic active versus the rim of inactive MS lesions, or the PL-NAWM of chronic active versus the PL-NAWM of inactive MS lesions.

Sample Preparation and Microarray Hybridization

The Low Input Quick Amp Labeling Kit (Agilent Technologies) was used for sample amplification and fluorescent labeling according to manufacturer's instructions. Briefly, 100 ng experimental RNA input and 50 ng reference pool RNA input was used for linear amplification and fluorescent labeling. The reference pool RNA was extracted from snap-frozen tissue dissected from a diversity of anatomical regions from control and MS brains, including MS lesions and NAWM, as well from tonsil. Experimental samples were labeled with Cy5-CTP, and the reference pool was labeled with Cy3-CTP (Perkin Elmer, Waltham, MA, USA). The cRNA samples were purified using RNeasy mini columns (Qiagen), and quantity and labeling efficiency (specific activity) was determined on a NanoDrop.

Prior to hybridization, 825 ng Cy3- and Cy5-labeled cRNA samples were fragmented by 30 min incubation at 60°C in 1× fragmentation buffer (Agilent Technologies). Each time, one Cy5-labeled experimental sample and one Cy3-labeled reference pool sample were hybridized to an Agilent Human Gene Expression 4 × 44K v2 Microarray (Part Number G4845A) for 17 h at 65°C in a rotating hybridization chamber. Arrays were washed in 6× saline sodium phosphate-EDTA (SSPE)/0.005% *N*-lauroylsarcosine (Sigma-Aldrich, St. Louis, MO, USA) for 5 min, then in 0.06× SSPE/0.005% *N*-lauroylsarcosine for 1 min, and finally in acetonitrile (Sigma-Aldrich) for 30 s. After drying in a nitrogen flow, arrays were scanned using an Agilent DNA Microarray Scanner at 5 mm resolution and 100% photomultiplier tube setting. Microarray scans were quantified using Agilent Feature Extraction software (version 9.5.3.1).

Microarray Normalization and Single Gene Analysis

Common reference cRNA was co-hybridized to every microarray slide to allow for accurate comparison of expression levels across different cDNA microarray experiments. In this way, a ratio between the experimental and reference material could be calculated for every spot, and expression levels across different hybridizations could be compared. Raw expression data were imported into the R statistical processing environment using the LIMMA package in Bioconductor.[2] All features for which one or more foreground measurements were flagged as saturated or as a non-uniformity outlier by the feature extraction software were excluded from further analysis. As overall background levels were very low, no background correction was performed.

Data within an array were normalized using *loess* (LIMMA), which was followed by a between-array normalization using the *Gquantile* algorithm in LIMMA. Subsequently, for probes that mapped to the same gene, the average *M*- and *A*-value of those probes were used for further analyses.

Differential gene expression was assessed using a single channel analysis on the M-values using Bayesian statistics in LIMMA. Three contrasts were investigated: (I) chronic active rim vs. inactive rim, (II) chronic active PL-NAWM vs. inactive PL-NAWM, and (III) chronic active PL-NAWM vs. control. Correction for multiple testing was performed with the Benjamini–Hochberg algorithm. Genes with a p-value <0.05 were considered significant.

Cluster Analysis of Gene Expression Data in Different Stages of Lesion Activity

In order to follow the expression of individual transcripts in different presumed subsequent stages of lesion activity and demyelination, expression profiles were constructed from regions that represent no pathology (control NAWM), the early events in demyelination (PL-NAWM around chronic active MS lesions), fully active demyelination (rim of chronic active MS lesions), halt of demyelination (rim of inactive MS lesions), and absence or suppression of early demyelination (PL-NAWM of inactive lesions, **Figure 1**). The NIA Array Analysis software was used to find these clusters of genes showing the same expression pattern across the different subareas studied (15). The intensity values of all genes were used as input. The NIA Array Analysis software

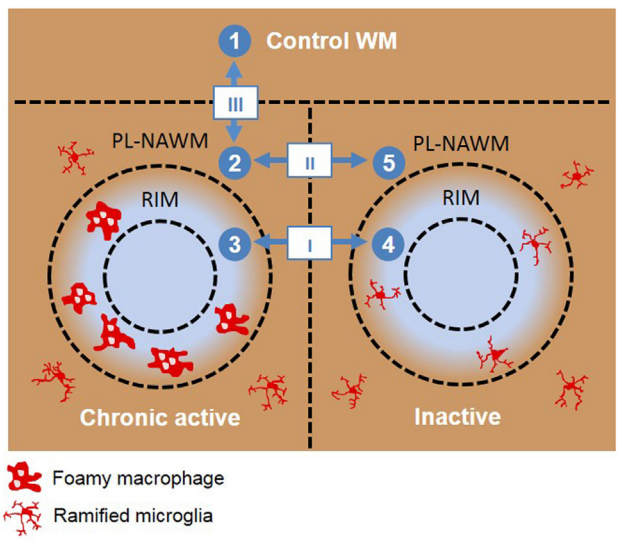

FIGURE 1 | Schematic overview of the different microarray analyses done. Roman numbers indicate direct comparisons. (I) chronic active rim vs. inactive rim, (II) chronic active peri-lesional (PL)-normal appearing white matter (NAWM) vs. inactive PL-NAWM, and (III) control vs. chronic active PL-NAWM. Arabic numbers indicate the sequence used for cluster analysis. (1) Control white matter (WM), (2) chronic active PL-NAWM, (3) chronic active rim, (4) inactive rim, (5) inactive PL-NAWM.

[2]http://www.bioconductor.org.

uses ANOVA (with error variance averaging and Benjamini correction for false discovery rate) to test statistical significant genes. Only significant genes were displayed.

Gene Ontology Overrepresentation Analysis

The overrepresentation of specific GO terms within the different clusters was analyzed using GOstat with the goa_human database (minimum path length of 3 and Benjamini correction for false discovery rate). All statistical significant genes per cluster were used as input, and all genes measured on the array were used as the background set of genes.

cDNA Synthesis and qPCR

Reverse transcription was performed in a reaction mixture of 10 µl containing 100 ng RNA and gDNA Wipeout Buffer, incubated for 2 min at 42°C, and Quantiscript® Reverse Transcriptase, Quantiscript Buffer, and room temperature (RT) Primer Mix (Qiagen Benelux, Venlo, The Netherlands), incubated for 15 min at 42°C. RT transcriptase was inactivated by incubation for 3 min at 95°C.

Primer pairs for real-time qPCR were designed using the NCBI primer basic local alignment search tool; see Table S2 in Supplementary Material for the primer pairs used in this study. Specificity was tested on cDNA derived from brain or laser dissection microscopy-isolated test brain tissue of MS donors and control donors by assessment of the dissociation curve and PCR product, as determined by size fractionation on an 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis gel.

Quantitative polymerase chain reaction reactions were performed with SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) with samples containing equal cDNA concentrations of 2–3.5 ng total RNA per reaction. Analysis was performed according to the manufacturer's protocol and the ABI Prism 7300 Sequence Detection System (Applied Biosystems). Target genes were normalized to the geometric mean of glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), tubulin α (*TUBA1A*), or elongation factor 1 alpha (*EEF1A1*) mRNA expression, which did not differ significantly between the different groups studied. Fold differences were calculated using the $2^{-\Delta\Delta CT}$ method (16).

Immunohistochemistry

Tissue of donors used for detection of protein expression are displayed in **Table 1**. For CHIT1, frozen sections (20 µm) of both active and inactive MS lesions and control tissue were fixed for 20 min in 4% paraformaldehyde. For GPNMB, OLR1, and ANO4, paraffin-embedded sections (6 µm) of both active and inactive MS lesions and control tissue were deparaffinized with xylene and rehydrated, and antigen retrieval was performed by incubation in tris-buffered saline (TBS) for GPNMB and OLR1 and in citrate buffer at pH6 for ANO4 (microwave, 10 min at 700 W). Aspecific binding was blocked by incubation in 10% normal horse serum (NHS) for 30 min at RT, followed by incubation with primary antibodies directed at CHIT1 (NBP1-84490, 1:20; Novus Biologicals, Abingdon, UK), GPNMB (MAB15501, 1:200; R&D Systems, Oxon, UK), OLR1 (H00004973-D01, 1:500; Abnova, Taoyuan City, Taiwan), or ANO4 (19488-1-AP, 1:50; Proteintech, Manchester, UK) diluted in incubation buffer (0.25% gelatin and 0.5% Triton-X in TBS, pH 7.6), for 1 h at RT. Immunoreactivity was visualized by using avidin–biotin complex (Vector PK-6100, Burlingame, CA, USA), followed by diaminobenzidine chromogenic substrate system (EnVision, DAKO) for CHIT1 or immediately by using the EnVision detection system (Dako) for GPNMB and OLR1. Sections were counterstained by 0.025% cresyl violet and embedded in Entellan. Immunoreactivity was examined using a Zeiss Axioskop 9801 light microscope (Zeiss, Oberkochen, Germany).

Cell Culture and Gene Silencing

The human monocytic cell line THP-1 was cultured in Roswell Park Memorial Institute (RPMI) glutaMAX medium containing 10% fetal calf serum and 1% penicillin/streptomycin. For flow cytometric or PCR analysis, cells were cultured in plates coated with poly(2-hydroxyethyl methacrylate), otherwise known as hydron (Sigma-Aldrich), to prevent adherence. For immunocytochemistry, cells were cultured on glass coverslips. Cells were differentiated into macrophage-like cells by stimulation with 160 nM phorbol 12-myristate 13-acetate (PMA) for 24 h, followed by another 24 h culture in normal medium. To measure unlabeled myelin uptake over time, cells were stimulated with 8 nM PMA for 48 h, followed by 5 days culture in normal medium.

Gene silencing was performed using locked nucleic acid (LNA) oligonucleotides, designed using the siDesign center of Thermo Scientific and synthesized by Santaris Pharma A/S (Hørsholm, Denmark). The following oligonucleotide sequences and concentrations were used: *MSR1* (CCCGTGAGACTTTGAG; 2 µM), *CXCL16* (AGTGAGCTCTTTGTCC; 5 µM), *OLR1* (CTCATTCAGCTTCCGA; 2.5 µM), and *CD68* (AACTGAAGC TCTGCCC; 2.5 µM). The 16-mers contained three LNA moieties at both termini (underlined). Oligonucleotide uptake was achieved without any additives, through a process called gymnosis (17). Differentiated cells were incubated with the oligonucleotides for 6 days before myelin uptake was tested. For inhibition of a broad spectrum of scavenger receptors, cells were pre-incubated with 100, 500, or 1,000 µg/ml fucoidan (Sigma, Zwijndrecht, The Netherlands) for 45 min before myelin was added.

Phagocytosis Assay

Myelin was isolated from the myelin-containing fraction of post-mortem human brain tissue collected after Percoll gradient separation. Unlabeled myelin was used to measure myelin uptake over time, and myelin stained with the pH sensitive dye pHrodo red (Invitrogen) was used for gene silencing experiments to visualize uptake in the lysosomal compartment, as described recently (18).

Free floating THP-1 macrophages were incubated with 12.5 µg pHrodo-labeled MS or control myelin per 80,000 cells for 24 h. After incubation, the cells were collected and washed in cold phosphate-buffered saline (PBS) with 1% bovine serum albumin for quantification of myelin uptake by flow cytometry after gene silencing. Expression of *CHIT1* and *GPNMB* was determined after incubation with unlabeled 12.5 µg MS or control myelin for 1, 2, or 5 days in duplo. After 5 days, the medium was refreshed

and cells were incubated for 80 more hours in normal medium. Harvested cells were stored in TRIsure and gene expression of *CHIT1* and *GPNMB* was analyzed by qRT-PCR.

For flow cytometric analysis, cells were incubated with the viability dye eFluor 780 (eBiosciences; 1:2,000) for 30 min on ice. Uptake of pHrodo-labeled myelin was measured on a FACSCanto machine (BD Biosciences) and analyzed using FlowJo 7.6 software (Figure S1 in Supplementary Material). Phagocytosis was expressed as percentage of live cells that took up myelin and as geomean fluorescence intensity of the pHrodo signal indicating the total amount of myelin phagocytosed.

For immunocytochemical analysis, cells on glass coverslips were fixed in 4% paraformaldehyde for 15 min and washed with PBS. Aspecific binding was blocked by incubation in 10% NHS for 30 min at RT, followed by incubation with the primary antibody directed at MSR1 (MAB1716, 1:100; Abnova), diluted in incubation buffer (0.25% gelatin and 0.5% Triton-X in TBS pH 7.6), o/n at 4°C. The next day, cells were washed and incubated with the fluorescently labeled secondary antibody (donkey anti-mouse Cy3 conjugated antibody, 1:1,000; Millipore) in incubation buffer with Hoechst 1:1,000 for nuclear staining for 1 h at RT. Coverslips were then washed in PBS and demineralized water and embedded in mounting medium (0.605 g Tris pH 8.5, 12.5 ml glycerol 100%, and 5 g Mowiol; EMD Chemicals, Gibbstown, NJ, USA).

Fluorescent images were taken on an Axiovert microscope (Zeiss) with Neoplanfluor objectives using an Exi Aqua Bioimaging microscopy camera (QImaging, Surray, BC, Canada) and ImagePro software (MediaCybernetics, Bethesda, MD, USA).

Statistical Analysis

Statistical analysis of qPCR validation and cell culture experiments was performed using GraphPad Prism version 6 software (GraphPad Inc., La Jolla, CA, USA). The non-parametric Kruskal–Wallis test, followed by *post hoc* comparisons (Mann–Whitney *U* test) was performed to assess the regulation of genes of interest and the effect of gene silencing and addition of fucoidan. Differences between THP-1 cells incubated with MS, control, or no myelin over time were assessed with One-way ANOVA, followed by Tukey's multiple comparisons test. p values <0.05 were considered significant.

RESULTS

Identification of Genes That Show Significantly Altered Expression in Different MS Lesion Subregions

Using laser-based microdissection, we isolated the rim and PL region of and around chronic active and inactive MS lesions from 15 MS patients and WM of 10 matched control subjects and analyzed differences in gene expression using Agilent Human Gene Expression 4 × 44K v2 microarrays. The following comparisons were made: I. chronic active rim vs. inactive rim, (II) chronic active PL-NAWM vs. inactive PL-NAWM, and (III) chronic active PL-NAWM vs. control WM (**Figure 1**; Roman numbers). In comparison I, we expected to find genes involved in either active demyelination (upregulated in chronic active rim) or cessation of demyelination (upregulated in inactive rim). Genes in comparison II were expected to be involved in early demyelination and expansion of lesions (upregulated in chronic active PL-NAWM) or the prevention and exhaustion of lesion expansion (upregulated in inactive PL-NAWM). Finally, comparison III was expected to show genes involved in initial lesion onset (either protective genes that are downregulated or inflammatory or phagocytic genes that are upregulated in chronic active PL-NAWM). This resulted in a total of 1,251 significantly regulated genes in comparison I, 587 genes in comparison II, and 3,434 genes in comparison III, with a p-value <0.05. For an overview of the top 50 significantly upregulated and downregulated genes per comparison, see **Figure 2**; Tables S3A–F in Supplementary Material.

Cluster Analysis

A cluster analysis was performed using the NIA Microarray Analysis Software to visualize gene expression during the presumed, subsequent stages of MS lesion activity and halt. The following sequence was chosen as input: (1) control WM, (2) chronic active PL-NAWM, where initial events in demyelination may be present in case the chronic active lesion was expanding, (3) chronic active rim, where active demyelination is ongoing, (4) inactive rim, where earlier demyelination has ceased, and (5) inactive PL-NAWM, where lesion progression has stopped (**Figure 1**; Arabic numbers). This gene cluster analysis revealed six specific patterns of gene expression between the subgroups tested (**Figure 3**). Some genes were generally expressed lower (cluster 1) or higher (cluster 2) in all lesion subregions, compared to control tissue. The other four patterns followed the presumed sequence of MS lesion development with a peak of gene expression around chronic active lesions (cluster 3), a peak of gene expression in the rim of chronic active lesions (cluster 4), low gene activity around active rims, but high expression in active rims and (peri)-rims of inactive lesions (cluster 5), and high gene expression in and around inactive lesions, but low activity in active rims (cluster 6).

The GO of the specific gene expression clusters was analyzed by GOstat (19) (Table S4 in Supplementary Material). The GO shows which gene functions are overrepresented within the groups studied, compared to all genes measured on the microarray. The most robust associations (i.e., with many GO terms changed) were found in cluster 2 and 4. In cluster 2, with overall high expression in MS, genes involved in immune functions were overrepresented, which is in line with MS being an inflammatory disease. In cluster 4, with highest expression in the active rim, genes involved in immune response/antigen presentation and cellular compartmentation, e.g., membrane and lysosome, were overrepresented, corresponding with the process of demyelination. In cluster 1, cluster 5, and cluster 6, associations were less robust. In cluster 1, with overall low expression in MS, various cellular functions linked with homeostasis were overrepresented. In cluster 5, with high expression in active rims and (peri)-rims of inactive lesions, but low activity in peri-rims of chronic active lesions, genes involved in extracellular matrix and/or collagen synthesis were overrepresented. In cluster 6, with high expression in and around inactive lesions, but low activity in active rims,

Comparison I: chronic active rim vs inactive rim				Comparison II: chronic active PL-NAWM vs inactive PL-NAWM				Comparison III: chronic active PL-NAWM vs control			
Gene symbol	FC	FC	Gene symbol	Gene symbol	FC	FC	Gene symbol	Gene symbol	FC	FC	Gene symbol
CHIT1	10.2	0.3	HBB	**NPY**	5.9	0.3	LOC100289290	**GPNMB**	7.1	0.1	AL050203
GPNMB	8.0	0.3	HBD	GABRA1	3.9	0.3	CRLF1	SERPINA3	7.0	0.1	DEFA3
CCL18	6.8	0.3	CRLF1	SYNPR	3.8	0.3	IGJ	APOC1	6.3	0.2	LOC100132168
ACP5	6.4	0.3	ABCA6	NPTX1	3.7	0.3	ABCA6	VIM	5.1	0.2	MYH7B
PKD2L1	5.7	0.3	KANK4	VSNL1	3.7	0.3	**KANK4**	**MSR1**	4.8	0.2	ENST00000443002
HLA-DQA1	4.7	0.4	RNU2-2	OPALIN	3.6	0.4	ENST00000392423	CD44	4.5	0.2	CPAMD8
FCGR2B	4.6	0.4	DHCR24	SYN2	3.5	0.4	C21orf130	**OLR1**	4.2	0.3	YJEFN3
CFD	4.5	0.4	TSPAN8	SNAP25	3.4	0.4	ARRDC4	PALLD	4.1	0.3	ATPGD1
APOC1	4.4	0.4	ENST00000392423	CREG2	3.3	0.4	ENST00000313339	CHI3L1	4.0	0.3	CIRBP
CXCR4	4.1	0.5	SQLE	GABRB2	3.2	0.4	TSPAN8	ANXA1	3.9	0.3	KCNQ2
PLA2G7	4.0	0.5	ENST00000313339	SYT4	3.1	0.4	TTN	EMP1	3.8	0.3	A_33_P3253832
HS3ST2	4.0	0.5	RBM11	SYT13	3.1	0.4	ARMC3	S100A10	3.8	0.3	ABCA2
IFI30	3.6	0.5	RNU1-5	* **NCAN**	2.7	0.5	RBM11	RFTN1	3.6	0.3	CITED4
CAPG	3.2	0.5	FSTL5	FGF13	2.6	0.5	FSTL5	IFI30	3.5	0.3	UNC84B
RAB42	3.0	0.5	RMRP	GDA	2.6	0.5	DHCR24	ANO6	3.5	0.3	LOC283999
MS4A7	3.0	0.5	WIF1	SV2B	2.5	0.5	C15orf51	CP	3.5	0.3	LIPE
ITGAX	2.9	0.5	TTN	CADPS	2.5	0.5	HYDIN	ABCA1	3.4	0.3	ENST00000369123
AMICA1	2.9	0.5	RELN	BASP1	2.5	0.5	ANKRD20B	FPR3	3.3	0.3	CERCAM
DENND2D	2.9	0.5	LOC728449	MAL2	2.5	0.5	POU2AF1	PLA2G7	3.3	0.3	ENST00000293201
OLR1	2.9	0.5	TNFRSF21	GNLY	2.5	0.5	MTUS1	CD84	3.3	0.3	LOC349114
ALOX5AP	2.8	0.5	LOC100133402	KCNC2	2.5	0.5	PPEF1	TGFB2	3.1	0.3	KLC2
FGR	2.8	0.5	C21orf130	KCNMA1	2.4	0.5	MBOAT1	FRMD3	3.1	0.3	ENST00000405068
CD68	2.8	0.5	LOC100290344	CDH18	2.4	0.5	RELL1	S100A6	3.1	0.3	DNAJB2
IRF5	2.8	0.5	PDE11A	TNFRSF12A	2.3	0.5	DPYSL5	NSL1	3.0	0.3	ADORA1
MSR1	2.8	0.5	SNORA28	RGS7BP	2.3	0.5	LOC728449	LGALS3	3.0	0.3	LOC340335
ASCL2	2.8	0.5	ANKRD20B	CBLN2	2.3	0.5	HN1L	SAMSN1	3.0	0.3	CCDC85B
HLA-DMB	2.8	0.5	LDLR	ENC1	2.2	0.5	TNFRSF21	MS4A7	3.0	0.3	A_33_P3209176
SLC7A7	2.8	0.5	ALAS2	PRICKLE1	2.2	0.5	ANKRD18A	COLEC12	2.9	0.3	ENST00000371623
TNFAIP2	2.7	0.5	KLK6	MRAP2	2.2	0.5	SNX24	DTNA	2.9	0.3	PCBP4
BCL2A1	2.7	0.5	RAPGEF5	HOPX	2.2	0.6	SPARC	FGF2	2.9	0.3	STMN4
HLA-DRB5	2.7	0.5	MBP	ELMOD1	2.2	0.6	GOLGA8E	NCF2	2.9	0.3	CMTM5
ADORA3	2.7	0.5	F5	FGF12	2.2	0.6	LOC283481	SPP1	2.9	0.3	TMEM63A
SLC47A1	2.7	0.5	HAPLN2	CHI3L1	2.1	0.6	AK058117	MAFB	2.8	0.3	DAO
MX2	2.7	0.5	ABCA8	NCEH1	2.1	0.6	ENST00000423618	SORBS1	2.8	0.3	HMX1
S100A11	2.6	0.5	NIPAL4	GABRB3	2.1	0.6	TTC25	DST	2.8	0.3	A_33_P3277805
NCF2	2.6	0.5	CDKN1C	CD83	2.1	0.6	LOC645321	PTPRC	2.8	0.3	LTBP3
CD83	2.6	0.5	GDF10	ISG15	2.1	0.6	MRO	DAAM1	2.8	0.3	DOHH
FCGR2C	2.6	0.5	MOBP	LRRN3	2.1	0.6	KLK6	DDR2	2.8	0.3	FTCD
ENST00000424686	2.6	0.5	HN1L	ALOX5AP	2.1	0.6	LOC284232	TRIM34	2.7	0.3	ST3GAL4
HLA-DQA2	2.6	0.5	ENPP6	ADORA3	2.1	0.6	RND2	S1PR3	2.7	0.3	FLJ45445
SIGLEC8	2.6	0.5	LOC283713	**OLR1**	2.1	0.6	LRAT	DENND2D	2.7	0.3	PAQR6
ENST00000412049	2.6	0.5	DPYSL5	* **ANO4**	2.1	0.6	NIPAL4	TPST1	2.6	0.3	tcag7.907
CCRL2	2.5	0.5	INSIG1	GAP43	2.1	0.6	DNAJC15	MAN2A1	2.6	0.3	PNPLA7
MS4A4A	2.5	0.6	LOC100008587	GFRA2	2.1	0.6	CCDC11	CNN3	2.6	0.3	LONP1
FCER1G	2.5	0.6	TMEM125	TMEM233	2.1	0.6	HAPLN2	MAN1C1	2.6	0.3	SBF1
HPSE	2.5	0.6	TMEM144	MX1	2.0	0.6	OFD1	CCRL2	2.6	0.3	ENST00000401999
LAPTM5	2.5	0.6	SPOCK3	CNR1	2.0	0.6	MAP4	EVI2B	2.5	0.3	HES6
LSP1	2.5	0.6	RHBDL2	BEX1	2.0	0.6	CFH	LIMS1	2.5	0.3	AGAP3
HLA-DMA	2.5	0.6	ERMN	GPR98	2.0	0.6	DNAH12	GPR65	2.5	0.3	ATP6V0E2
KLHL6	2.5	0.6	DMBT1	IFI30	2.0	0.6	MOBKL2B	SLC5A3	2.5	0.3	MVD

FIGURE 2 | Top 50 significantly upregulated and downregulated genes in multiple sclerosis lesion subregions. For a description of the comparisons, see **Figure 1** and text. Upregulated genes are indicated in red and downregulated genes are indicated in blue. Genes colored and marked in bold have been selected for further investigation. Genes expressed in cluster 3 are marked by an asterisk. Further details on the genes and *p* values are provided in Tables S3A–F in Supplementary Material. FC, fold change.

sterol biosynthesis was overrepresented. The number of genes that fell within cluster 3 ($n = 3$) was too low for the GO analysis. The GO also showed a shift in the expression location of overrepresented genes from the plasma membrane to the lysosomal membrane from comparison II to comparison I (data not shown). Furthermore, processes involved in lipid metabolism were overrepresented in comparison I (data not shown).

Selection of Genes of Interest

Genes of interest were selected based on their regulation between the subgroups (direct comparisons) and their expression pattern in the cluster analysis, with a specific interest of genes regulated peri-lesionally around chronic active lesions.

CHIT1 (chitinase 1) *GPNMB* (glycoprotein non-metastatic melanoma protein B), and *CCL18* (C–C motif chemokine

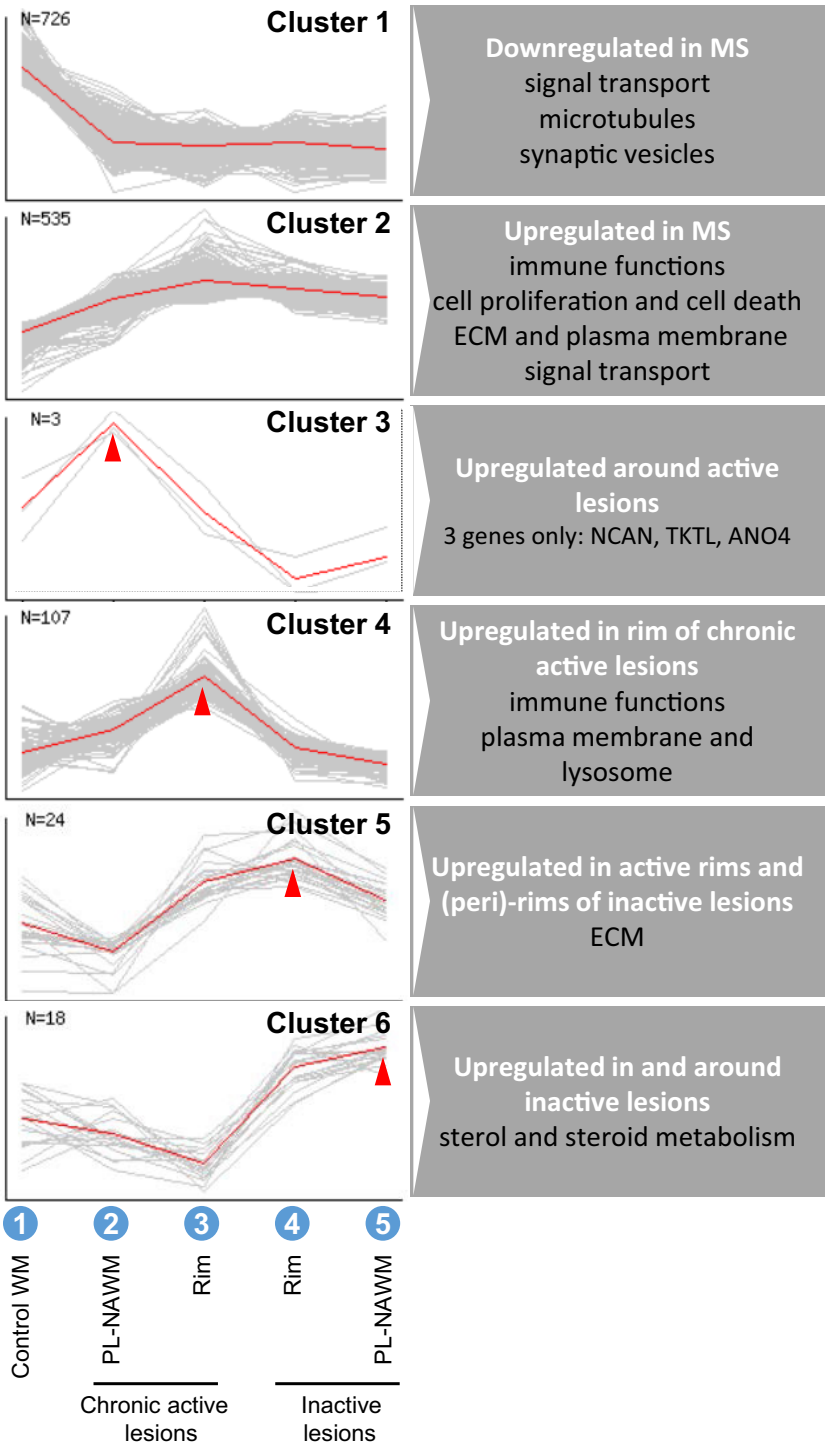

FIGURE 3 | Cluster analysis of gene expression in and around multiple sclerosis (MS) lesions. Analysis was done with the sequence: (1) control white matter (WM), (2) chronic active peri-lesional (PL)-normal appearing white matter (NAWM), (3) chronic active rim, (4) inactive rim, (5) inactive PL-NAWM (also shown in **Figure 1**), which resulted in six different expression patterns, representing overall differences between control and MS (clusters 1 and 2), specific upregulation around expanding chronic active lesions (cluster 3), specific upregulation in active rims (cluster 4), upregulation in active rims and (peri)-rims of inactive lesions (cluster 5), or upregulation in and around inactive lesions (cluster 6). *N* indicates the number of significantly regulated genes within a cluster.

ligand 18) were the most upregulated genes in comparison I. *CHIT1* showed the highest expression in the rim of chronic active MS lesions, with a 10.2-fold change, compared to the expression in the rim of inactive MS lesions (**Figure 2**). *CHIT1* was also slightly upregulated in the PL-NAWM of chronic active MS lesions, compared to the PL-NAWM of inactive MS lesions (fold

change of 2.0). *GPNMB* and *CCL18* showed a peak in expression in the rim of chronic active MS lesions, compared to the rim of inactive MS lesions, with a fold change of 8.0 and 6.8, respectively (**Figure 2**). Moreover, *GPNMB* was most highly induced around chronic active lesions compared to control tissue (fold change of 7.1) in comparison III.

Myelin recognition and uptake requires the presence of dedicated receptors at the surface of microglia/macrophages. We previously reported selective upregulation of scavenger receptors in and around demyelinating areas in MS (14). Congruently, genes upregulated in the rim of chronic active MS lesions compared to the rim of inactive MS lesions (comparison I) included the scavenger receptors *OLR1* [oxidized low-density lipoprotein receptor 1, also known as lectin-type oxidized LDL receptor 1 (LOX-1)], *CD68* (cluster of differentiation 68), *MSR1* [macrophage scavenger receptor 1, also known as scavenger receptor AI/II (SR-AI/II)], and *CXCL16* (C-X-C motif chemokine ligand 16) (fold change of 2.8–1.8) (**Figure 2**). *OLR1*, *CD68*, and *MSR1* were also upregulated in the PL-NAWM of chronic active MS lesions, compared to the PL-NAWM of inactive MS lesions and to control tissue (comparisons II and III), indicating that these molecules may be involved in initial demyelination. All scavenger receptors showed the highest expression in the rim of chronic active MS lesions.

Three other molecules of interest were *CXCR4* (C-X-C motif chemokine receptor 4), *NPY* (neuropeptide Y), and *KANK4* (KN motif and ankyrin repeat domains 4). The chemokine receptor *CXCR4* was upregulated in comparison I (fold change of 4.1) (**Figure 2**). The neurotransmitter *NPY* had the highest fold change in comparison II (fold change of 5.9) (**Figure 2**). Expression was highest in control tissue and PL-NAWM of chronic active MS lesions, lower in the rim of chronic active MS lesions, and lowest in inactive MS lesions (data not shown). The cytoplasmic protein *KANK4* had the highest *p*-value and was downregulated in the PL-NAWM of chronic active MS lesions compared to the PL-NAWM inactive lesions (comparison II; fold change of 0.3) (**Figure 2**). Expression was highest in inactive MS lesions and lower in control tissue and in chronic active MS lesions (data not shown).

Finally, *NCAN* (neurocan), *TKTL1* (transketolase-like 1), and *ANO4* (anoctamin 4) were included because these were the only genes in cluster 3, with a specific peak in expression in the PL-NAWM of chronic active MS lesions (**Figure 3**). *NCAN* and *ANO4* are also upregulated in the PL-NAWM of chronic active MS lesions compared to the PL-NAWM of inactive lesions (comparison II; fold change of 2.7 and 2.1) (**Figure 2**). Immunohistochemical staining showed more explicit ANO4 expression in the PL-NAWM of chronic active lesion compared to PL-NAWM of inactive MS lesion (Figure S2 in Supplementary Material).

Validation and Further Characterization of Genes of Interest

There was a significant difference of the RIN value of control tissue compared to all MS lesion subareas, which is not unexpected as the control tissue was dissected in the cryostat using a scalpel as opposed to laser-based microdissection of the MS tissue. This significant difference did not influence our conclusions, as microarray data were validated by qPCR (**Table 2**), which showed no effect of RIN value on expression levels when normalizing with housekeeping genes (20). There was no significant difference in RIN values between the different MS lesion areas.

Significant differences in gene expression were confirmed for *CHIT1*, *GPNMB*, *CCL18*, *KANK4*, *OLR1*, *CD68*, *MSR1*, and *CXCL16* in comparison I; for *NPY*, *OLR1*, *CD68*, and *MSR1* in comparison II; and for *GPNMB*, *OLR1*, *CD68*, and *MSR1* in comparison III. Some genes that showed no significant difference with microarray, did show a significant different with qPCR, e.g., *NPY* in comparison I, *GPNMB* and *CXCL16* in comparison II, and *CHIT1* and *KANK4* in comparison III. *CXCR4* showed no significant difference when validated with qPCR.

The expression pattern for most genes was similar with qPCR as compared to the microarray. *CHIT1*, *GPNMB*, *CCL18*, *CXCR4*,

TABLE 2 | Selected genes of interest.

Gene symbol	Comparison I: chronic active rim vs inactive rim				Comparison II: chronic active PL-NAWM vs inactive PL-NAWM				Comparison III: chronic active PL-NAWM vs control			
	Microarray		qPCR		Microarray		qPCR		Microarray		qPCR	
	p-Value	Fold change	*p*-Value	Fold change	*p*-Value	Fold change	*p*-Value	Fold change	*p*-Value	Fold change	*p*-Value	Fold change
CHIT1	1.07E−18	10.2	7.00E−03	36.0	4.28E−02	2.0	n.d.	n.d.	n.s.	n.a.	1.20E−03	5.8
GPNMB	3.61E−08	8.0	3.00E−04	12.4	n.s.	n.a.	2.20E−03	8.3	7.40E−06	7.1	3.10E−03	6.7
CCL18	2.13E−15	6.8	4.76E−02	66.7	n.s.	n.a.	n.s.	n.a.	n.s.	n.a.	n.s.	n.a.
OLR1	2.76E−04	2.9	5.90E−03	3.2	4.36E−02	2.1	6.00E−04	7.5	4.86E−06	4.2	2.00E−04	6.0
CD68	1.55E−07	2.8	6.00E−04	5.2	3.15E−02	1.7	3.00E−04	4.8	3.16E−02	2.2	4.00E−04	5.0
MSR1	2.09E−04	2.8	5.90E−03	4.0	4.18E−02	1.8	2.05E−02	5.3	1.64E−04	4.8	7.00E−04	7.3
CXCL16	4.99E−04	1.8	1.75E−02	2.9	n.s.	n.a.	9.30E−03	2.8	n.s.	n.a.	n.s.	n.a.
CXCR4	1.08E−04	4.1	n.s.	n.a.	n.s.	n.a.	n.s.	n.a.	n.s.	n.a.	n.s.	n.a.
NPY	n.s.	n.a.	4.01E−02	6.0	1.15E−03	5.9	4.10E−03	22.9	n.s.	n.a.	n.s.	n.a.
KANK4	7.36E−09	0.4	1.40E−02	0.2	5.65E−06	0.3	n.s.	n.a.	n.s.	n.a.	6.80E−03	0.2

n.a., not applicable; n.d., not determined; n.s., not significant; PL-NAWM, peri-lesional normal appearing white matter; qPCR, quantitative polymerase chain reaction.

OLR1, *CD68*, *MSR1*, and *CXCL16* all showed highest expression in the rim of chronic active MS lesions. *GPNMB, OLR1, CD68,* and *MSR1* were upregulated in comparison II and III, and *CXCL16* in comparison II. Expression of *NPY* was highest in control tissue and the PL-NAWM of chronic active MS lesions, lower in the rim of chronic active MS lesions, and lowest in inactive MS lesions. The expression pattern for *KANK4* was different with qPCR. Expression levels for *KANK4* peaked in inactive MS lesions with microarray, but showed a peak in control tissue with qPCR. Within MS subregions, *KANK4* expression was still highest in the rim of inactive MS lesions.

Expression of *CHIT1*, *GPNMB*, and *OLR1* was further investigated at the protein level by immunohistochemistry (**Figure 4**). CHIT1, GPNMB, and OLR1 were not detected in control tissue, but were clearly present in chronic active MS lesions in the gliotic center, the rim, and also the peri-rim, following the RNA expression pattern. In inactive MS lesions, only GPNMB expression was identified in the center of the lesion and the lesion rim. Protein

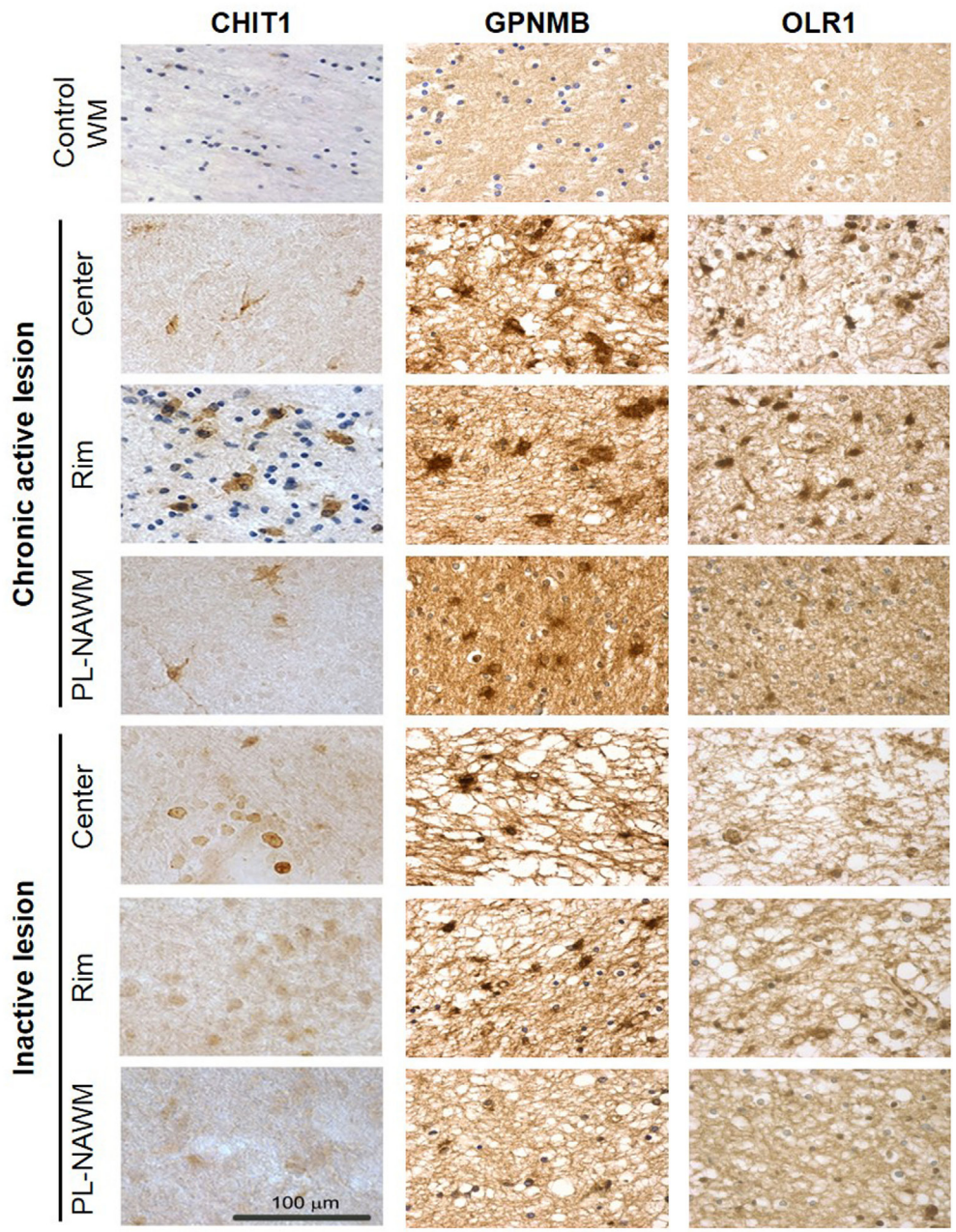

FIGURE 4 | Expression of CHIT1, GPNMB, and OLR1 in and around multiple sclerosis (MS) lesions. Protein expression of CHIT1, GPNMB, and OLR1 in control tissue and in the center, rim, and peri-lesional (PL)-normal appearing white matter (NAWM) of chronic active and inactive MS lesions determined by immunohistochemistry. Scale bar = 100 µm.

expression of CD68, MSR1, and CXCL16 in and around chronic active MS lesions has been described previously by us (14).

Notably, these three genes have also been linked with Gaucher disease, a lysosomal storage disorder (see Discussion) and may relate to the process of myelin ingestion by microglia/macrophages. We next tested whether expression of *CHIT1* and *GPNMB* is increased upon myelin uptake, as this has been demonstrated for *CCL18* (21). Differentiated THP-1 macrophages were exposed to myelin from MS or control donors for 5 days, followed by 3 days culture in normal medium (**Figure 5**). After 8 days in culture, cells cultured with MS myelin showed a significant increase in *CHIT1* expression ($p = 0.02$) and a trend toward more *GPNMB* ($p = 0.07$) expression, compared to no myelin uptake. Uptake of myelin from control donors and myelin ingestion over time did not show an altered expression pattern. To conclude, both *CHIT1* and *GPNMB* are highly expressed in the rim and peri-rim of chronic active lesions, likely due to the uptake of MS myelin by microglia/macrophages.

The Effect of Scavenger Receptor Knockdown on Myelin Phagocytosis *In Vitro*

As this study and our earlier work (14) revealed high expression of scavenger receptors in the rim as well as around chronic active MS lesions, we studied the role of *OLR1*, *CD68*, *MSR1*, and *CXCL16* in an *in vitro* myelin phagocytosis assay (**Figure 6**). Antisense oligonucleotides were developed that downregulate expression of these genes in the human macrophage cell line THP-1, confirmed by qPCR (**Figure 6A**). Immunocytochemistry confirmed the efficient knockdown of MSR1 (**Figure 6B**). Knockdown of *MSR1* and *CXCL16* significantly decreased the percentage of macrophages that phagocytosed myelin and the total myelin phagocytosed, compared to untreated cells (**Figure 6C**). Phagocytosis of myelin derived from control or MS tissue was similarly reduced by *MSR1* knockdown (**Figure 6D**). To block scavenger receptor function in a redundant manner, we also applied a broad pharmacological inhibitor (22, 23). Fucoidan showed a non-toxic, dose-dependent significant reduction of the percentage of macrophages that phagocytosed myelin and the total amount of myelin phagocytosed at 1,000 μg/ml ($p = 0.007$; **Figure 6E**). Upregulation of scavenger receptors in chronic active MS lesions thus likely contributes to demyelination.

DISCUSSION

In this study, we compared gene expression in and around chronic active MS lesions, inactive MS lesions, and control tissue to identify gene expression related to lesion activity and lesion halt. We found upregulation of genes involved in immune function, lipid binding, and lipid uptake in the active rim. This confirms the expectation, since in rims of chronic active lesions, inflammatory microglia/macrophage phagocytose myelin. Importantly, around chronic active MS lesions, genes involved in lipid binding and uptake also showed increased expression. This indicates early demyelination around chronic active lesions, showing that these lesions are indeed expanding. In addition, genes with a possible anti-inflammatory and/or neuroprotective function were upregulated in rims and around chronic active expanding lesions, possibly relating to the induction of endogenous protective mechanisms. Based on direct comparisons and cluster analysis, and with a specific focus on genes related to lesion activity and expansion, we identified several genes of interest: *CHIT1*, *GPNMB*, *CCL18*, *OLR1*, *CD68*, *MSR1*, *CXCL16*, *CXCR4*, *NPY*, *KANK4*, *NCAN*, *TKTL1*, and *ANO4*.

Altered Gene Expression in the Rim and PL-NAWM of Chronic Active and Inactive MS Lesions

To visualize gene expression during MS lesion progression, we used the set-up as shown in **Figure 1**, which was thought to best

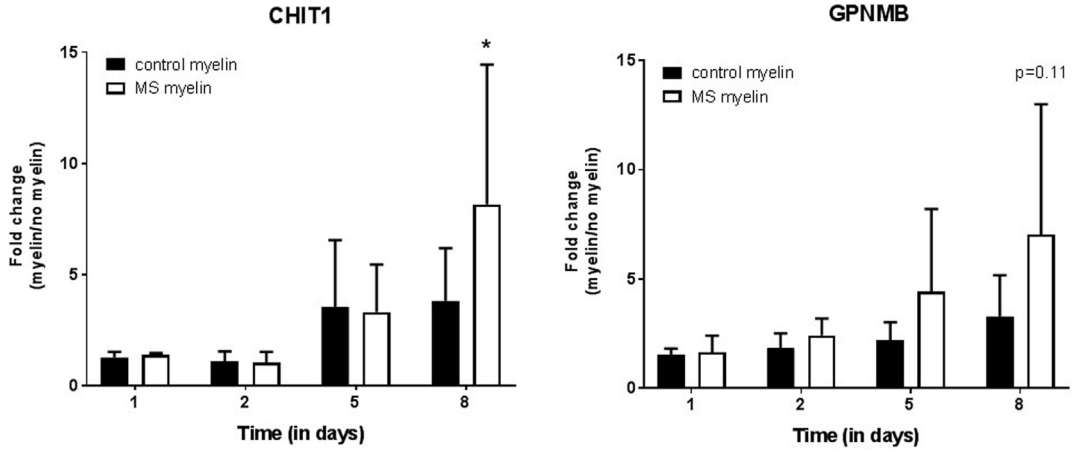

FIGURE 5 | Upregulation of *CHIT1* after uptake of multiple sclerosis (MS) myelin *in vitro*. At the mRNA level, both *CHIT1* ($p = 0.02$) and *GPNMB* ($p = 0.007$) are upregulated in THP-1 macrophages after incubation with myelin from MS donors for 5 days, followed by 3 days incubation in normal medium. Fold change from macrophages cultured without myelin, $n = 3$, $^*p < 0.05$.

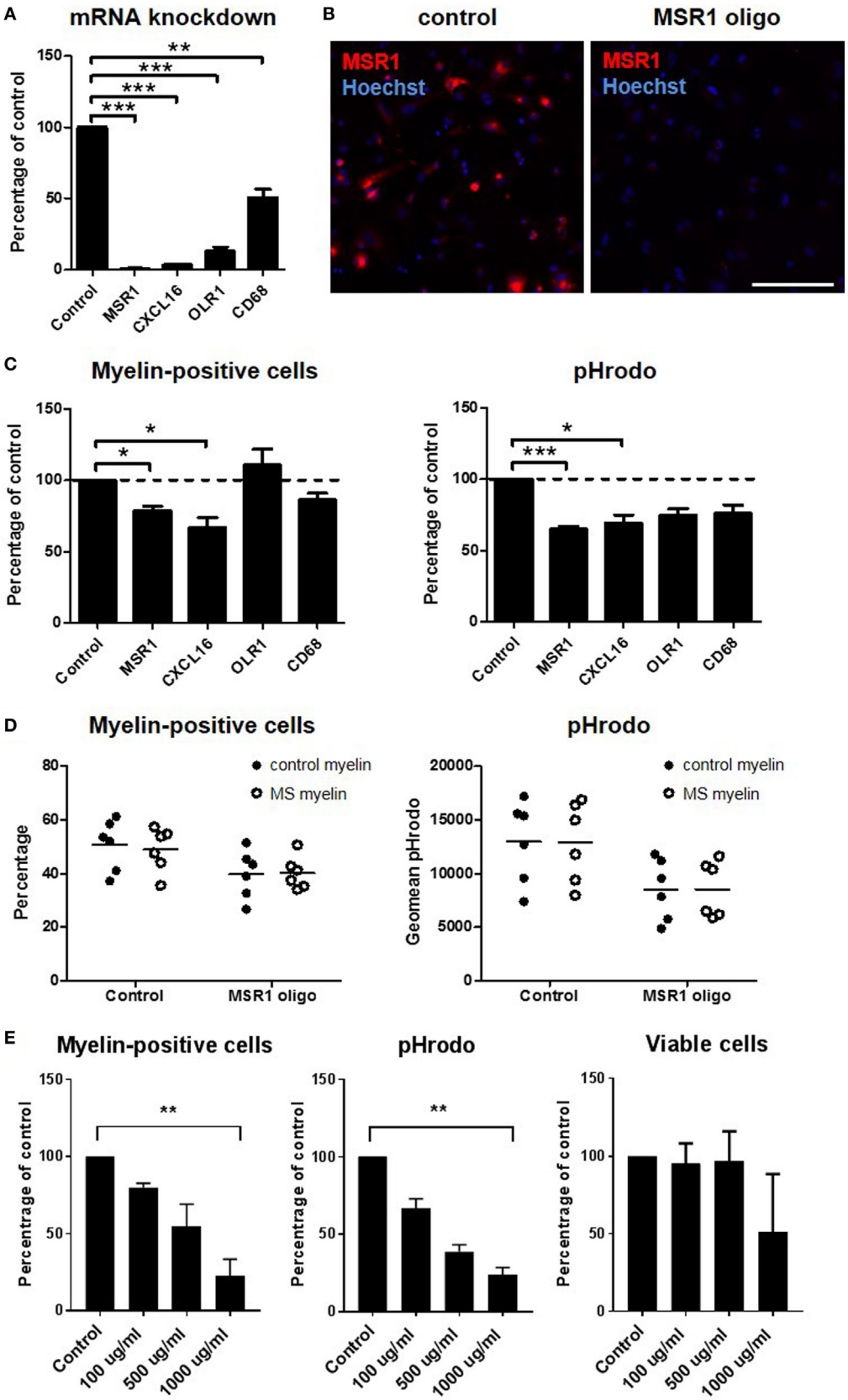

FIGURE 6 | Continued

FIGURE 6 | Functional role of scavenger receptors in myelin phagocytosis *in vitro*. *MSR1*, *CXCL16*, *OLR1*, and *CD68* were downregulated with antisense oligonucleotides in the human macrophage cell line THP-1. Silencing efficiency was determined on mRNA level with quantitative polymerase chain reaction **(A)** and on protein level with immunocytochemistry [**(B)**; only shown for MSR1]. Uptake of pHrodo-labeled myelin was validated by flow cytometry **(C)** and compared for myelin obtained from control and multiple sclerosis (MS) brain tissue **(D)**. The number of cells that had phagocytosed myelin, and the total amount of myelin phagocytosed (geomean pHrodo) were calculated. The number of independent experiments (*n*) was 6 (MSR1), 4 (CXCL16), 3 (OLR1), and 3 (CD68). **(E)** Fucoidan was used to inhibit a broad spectrum of scavenger receptors in THP-1 cells. Provided is the number of cells that had phagocytosed myelin, the total amount of myelin phagocytosed (geomean pHrodo), and the viability of cells at the time point of analysis ($n = 3$). Scale bar in panel **(B)** = 200 μm $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$.

resemble the sequence of events in MS lesion formation and progression. Cluster analysis (**Figure 3**) revealed six different expression patterns representing overall differences between control and MS (clusters 1 and 2), specific upregulation only around expanding, chronic active lesions (cluster 3), specific upregulation in active rims (cluster 4), upregulation in active rims and around inactive lesions, but low activity around active rims (cluster 5), or upregulation in and around inactive lesions, but low activity in active rims (cluster 6). Overrepresented genes within each cluster were detected by GO analysis (Table S4 in Supplementary Material). Clusters 2 and 4 showed genes involved in immune functions, which is expected as these genes peak in the rim of chronic active MS lesions. Not unexpected, genes involved in the lysosomal activity were overrepresented in cluster 4, corresponding with the process of demyelination. Genes involved in sterol and steroid metabolism were overrepresented in cluster 6, possibly indicating an attempt to repair damaged axons and myelin after active demyelination has diminished. In cluster 3, only three genes were regulated (discussed below).

The GO analysis also showed a shift in the expression location of overrepresented genes from the plasma membrane to the lysosomal membrane from comparison II to comparison I (data not shown). This is not surprising, as myelin first needs to be recognized and phagocytosed, before it can be processed in the lysosomes. Furthermore, processes involved in lipid metabolism were overrepresented in comparison I (data not shown), indicating that phagocytosed myelin is being processed.

Joint Upregulation of CHIT1, GPNMB, and CCL18 Is Mediated by Myelin Uptake

Our study identified *CHIT1*, *GPNMB*, and *CCL18* as top-3 upregulated genes in the rim of chronic active MS lesions, where foamy, myelin-accumulating microglia/macrophages are abundant. Interestingly, also in regions surrounding chronic active lesions, *CHIT1* and *GPNMB* were upregulated. Enhanced expression of *CCL18* in myelin-laden macrophages *in vitro* and in the rim and center of active MS lesions has been reported before (21). Here, we demonstrate a relation between the regulation of both, *CHIT1* and *GPNMB*, and myelin uptake. Both genes showed an increased expression in THP-1-derived macrophages after 8 days of incubation with MS myelin, and not with control myelin, suggesting that specifically myelin derived from MS donors induce *CHIT1* and *GPNMB* expression. Wheeler and colleagues already reported that the composition of MS myelin in NAWM is altered, compared to control myelin (24), and previously, we described that MS myelin is taken up more efficiently (18).

Of note, upregulation of *CHIT1* and *CCL18* in lipid-laden macrophages of Gaucher patients has long been known (25, 26). More recently, *CHIT1* has been described as a prognostic biomarker for early MS (27, 28), and soluble *GPNMB*, which is secreted from different cell types through a disintegrin and metalloproteinase 10 sheddase activity, was identified as a further biomarker of Gaucher disease (29, 30). Co-regulation of *CHIT1*, *GPNMB*, and *CCL18* strengthens the idea that lipid-accumulating Gaucher cells and myelin-phagocytosing microglia/macrophages share cellular characteristics (21), possibly related to lysosomal stress, a function overrepresented in active rims (cluster 4). The precise relationship between the induction of *CHIT1*, *GPNMB*, and *CCL18*, myelin processing, and lysosomal activities of microglia in MS warrants further investigation.

Functional studies have linked *CHIT1*, *GPNMB*, and *CCL18* primarily with the suppression of inflammation. Inhibition of *CHIT1* in a mouse macrophage cell line induced a pro-inflammatory phenotype *in vitro*, caused downregulation of *MSR1* and CD68, and decreased cholesterol uptake (31). Notably, *CHIT1* levels in the CSF reliably indicate microglial activation in clinical trials (32). *GPNMB* was upregulated in astrocytes and neurons in an animal model of amyotrophic lateral sclerosis, and secretion of the extracellular fragment by astrocytes had a neuroprotective effect (33). Another study showed that *GPNMB* was mainly expressed by macrophages/microglia in the rat brain and was upregulated in inflammatory conditions (34), acting as a negative regulator to prevent excessive immune responses (35). In line herewith, *Gpnmb*-deficient mice (DC-HIL$^{-/-}$) manifested exacerbated autoimmune encephalomyelitis (EAE) (36). Finally, *CCL18* recruited a subset of human regulatory T cells *in vitro*, which suppressed proliferation of effector T cells via interleukin-10 production (37). *CCL18* is also produced by macrophages that have ingested myelin and show an immunosuppressive phenotype (21). Suppressive effects on inflammation by *CHIT1*, *GPNMB*, and *CXCL18* in relation to demyelination in regions surrounding active MS lesions need to be elucidated.

Scavenger Receptors Upregulated in and around Chronic Active MS Lesions Mediate Myelin Uptake

Myelin uptake during demyelination likely depends on scavenger receptors. We found *OLR1*, *CD68*, *MSR1*, and *CXCL16* upregulated in all three comparisons (*CXCL16* only in comparison I and in comparison II with qPCR). Thus, these scavenger receptors are upregulated in chronic active rims, but also around chronic active lesions, indicating that they are involved in early demyelination. We cannot fully exclude some contamination of PL regions with rim tissue during laser dissection, but early demyelination around chronic active lesions is further indicated

by immunohistochemistry, showing upregulation of *CHIT1*, *GPNMB*, and *OLR1* in and around chronic active MS lesions. These results extends our earlier work, showing enhanced expression of *CD68*, *MSR1*, and *CXCL16* in and around chronic active MS lesions, compared to control tissue (14). *CD68* is a scavenger receptor predominantly expressed on the lysosomal membrane, but the small percentage expressed on the plasma membrane is capable of oxLDL phagocytosis (38, 39). SA-RI/II, encoded by *MSR1*, was shown to be directly involved in myelin uptake *in vitro* (40–42), and *Msr1*$^{-/-}$ mice showed less severe disease and reduced demyelination in the EAE model (43). *CXCL16* has a dual function as a scavenger receptor and as a chemokine in soluble form. Neutralizing antibodies against *CXCL16* delayed the onset and reduced the severity of EAE in mice (44). The soluble form is elevated in MS patients, compared to control subjects (45). This indicates that scavenger receptors could be actively involved in demyelination in MS. Furthermore, their upregulation in the PL-NAWM of chronic active MS lesions, might suggest they are involved in the initial stages of MS lesion development and progression.

The functional role of *OLR1, CD68, MSR1*, and *CXCL16* was studied in an *in vitro* myelin phagocytosis assay, using the human macrophage cell line THP-1. All genes were significantly downregulated, compared to untreated cells. Downregulation of *MSR1* and *CXCL16* resulted in a significant decrease in the number of myelin-phagocytosing cells and total myelin uptake. A direct role of *MSR1* in myelin phagocytosis in MS is consistent with earlier research (40–42) and supported by its expression in regions of active demyelination in MS brain tissue. Its upregulation in the PL-NAWM of chronic active MS lesions could indicate that phagocytosing cells in yet unaffected areas are already preparing for demyelination. Notably, pharmacological inhibition of a broad spectrum of scavenger receptors by fucoidan further reduced the number of phagocytosing macrophages and the total amount of myelin uptake, indicating that a combination of scavenger receptors contributes to the uptake of myelin.

Molecules Associated with Lesion Expansion

Our analysis identified many more genes regulated in and around chronic active and inactive MS lesions, which are potential targets to regulate lesion expansion. *NCAN*, *TKTL1*, and *ANO4* were of specific interest because they peaked at the PL-NAWM of chronic active MS lesions (cluster 3). Consistent with our findings, an upregulation of the extracellular matrix component *NCAN* in the rim and also slightly in the PL-NAWM of MS lesions has been reported earlier (46). Furthermore, *NCAN* is expressed by astrocytes and known to be upregulated after brain injury and modulates neuronal outgrowth (47). *TKTL1* is a transketolase expressed by mature oligodendrocytes in PL-NAWM of MS lesions and by oligodendrocyte precursors, reactive astrocytes, and macrophages in the rim of MS lesions (48) that has been postulated to prevent neurodegeneration by reducing the formation of advanced glycation end products and radicals (49). Upregulation of *TKTL1* might be an initial protective reaction to changes taking place in the PL-NAWM of MS lesions. Both *NCAN* and *TKTL1* might be important regulators in early axonal damage. Finally, the potential role of *ANO4*, a suggested Ca^{2+}-dependent lipid scramblase, in MS lesion development is unknown. In contrast to chronic active lesions, the PL-NAWM of inactive lesions show overrepresented genes involved in sterol biosynthesis, which might indicate an attempt to restore damaged tissue and myelin after active demyelination has diminished.

Both *KANK4* and *NPY* are regulated in the RIM and PL-NAWM of chronic active lesion, where *KANK4* was downregulated and *NPY* showed an upregulation. KANK family proteins are involved in the inhibition of actin stress fibers formation and cell motility [reviewed in Ref. (50)], but the exact function of *KANK4* and its role in MS lesions formation needs to be determined. In contrast to *KANK4*, the neurotransmitter *NPY* was upregulated in chronic active lesions. Application of *NPY* during EAE induction significantly suppressed clinical signs in DA rats (51) and in mice (52). *NPY* also inhibited disease and reduced inflammation when administered after the onset of EAE symptoms (53). Fc receptor-dependent phagocytosis of opsonized latex beads by lipopolysaccharide-stimulated microglia was reduced by *NPY* (54). Induction of *NPY* in and around acute MS lesions, might regulate myelin phagocytosis and immune suppression during early demyelination events.

Studying gene expression profiles associated with expansion of chronic active MS lesions is essential, since we recently showed that chronic active lesions highly correlate to disease progression (Luchetti et al., submitted). Taken together, we found changes in immune activation and lipid uptake in the rim of chronic active MS lesions. Genes related to lipid phagocytosis were also upregulated in the PL-NAWM of chronic active MS lesions, showing that chronic active lesions indeed expand. Importantly, potentially protective, anti-inflammatory genes were also upregulated in the (peri)-rim of chronic active MS lesions, suggesting a vain attempt to prevent lesion expansion and progression. We functionally confirmed the ability of the scavenger receptors *MSR1* and *CXCL16* to mediate myelin uptake. Our results pinpoint scavenger receptors as interesting targets to stop demyelination around chronic active lesions, which could block lesion expansion.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Netherlands Brain Bank (NBB, Amsterdam, The Netherlands). The NBB has obtained approval from the ethical medical committee of VU University medical center (VUmc, Amsterdam, The Netherlands) for all documents and procedures of brain banking, including the informed consent for the use of tissue and medical records for research purposes. Ethical, judicial, and anonymization strategies are all according to the published code of conduct for European brain banks (see for ethical approval, ethical documents and the ethical code of conduct for Brain banking: https://www.brainbank.nl/about-us/the-nbb/).

AUTHOR CONTRIBUTIONS

DH carried out tissue laser dissections, microarray hybridizations, gene expression analyses, immunohistochemical stainings

and drafted the manuscript. JS performed immunohistochemical stainings and phagocytosis experiments. MP performed phagocytosis experiments and helped with drafting of the manuscript. KB carried out gene expression analyses. KS isolated microglia and myelin. CE performed qPCRs and immunohistochemical stainings. EH participated in study coordination and data analysis. JH and IH were responsible for study design and coordination, data interpretation, and drafting of the manuscript. All authors have read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/articles/10.3389/fimmu.2017.01810/full#supplementary-material.

DATA AVAILABILITY

Microarray data have been uploaded in the Gene Expression Omnibus (GEO) database. The GEO accession number to access the complete dataset is: GSE108000.

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REVIEW
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NLR-Dependent Regulation of Inflammation in Multiple Sclerosis

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Multiple sclerosis (MS) is an autoimmune disease of the central nervous system (CNS) associated with inappropriate activation of lymphocytes, hyperinflammatory responses, demyelination, and neuronal damage. In the past decade, a number of biological immunomodulators have been developed that suppress the peripheral immune responses and slow down the progression of the disease. However, once the inflammation of the CNS has commenced, it can cause serious permanent neuronal damage. Therefore, there is a need for developing novel therapeutic approaches that control and regulate inflammatory responses within the CNS. Nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) are intracellular regulators of inflammation expressed by many cell types within the CNS. They redirect multiple signaling pathways initiated by pathogens and molecules released by injured tissues. NLR family members include positive regulators of inflammation, such as NLRP3 and NLRC4 and anti-inflammatory NLRs, such as NLRX1 and NLRP12. They exert immunomodulatory effect at the level of peripheral immune responses, including antigen recognition and lymphocyte activation and differentiation. Also, NLRs regulate tissue inflammatory responses. Understanding the molecular mechanisms that are placed at the crossroad of innate and adaptive immune responses, such as NLR-dependent pathways, could lead to the discovery of new therapeutic targets. In this review, we provide a summary of the role of NLRs in the pathogenesis of MS. We also summarize how anti-inflammatory NLRs regulate the immune response within the CNS. Finally, we speculate the therapeutic potential of targeting NLRs in MS.

Keywords: NLRs, multiple sclerosis, NLRP3, NLRP12, NLRX1, inflammation, astrocytes, microglia

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INTRODUCTION

Inflammation is a key component that accompanies the pathophysiology of all diseases (1). Multiple sclerosis (MS) is a neurodegenerative and demyelinating disease with a well-defined inflammatory component. Therefore, homeostatic processes that regulate inflammation may yield important insights into pathophysiology of MS. There are many definitions of inflammation with various levels of complexity. We define inflammation as an innate immune system-mediated process that is governed by the proinflammatory cytokines and chemokines, such as TNF-α, IL-1α, IL-1β, IL-6, IL-18, GM-SCF, IL-8, and MIP1a. As a result, the robust inflammatory

response is associated with the increased expression of proteins in enzymatic pathways, which leads to the release of cytotoxic molecules, including nitric oxide (NO), reactive oxygen species (ROS), prostaglandins, and an array of proteases. The fundamental role of inflammatory responses is to eliminate invading pathogens and to help an organism recover from tissue damage. Therefore, the immunological responses to infection or tissue injury are often associated with the release of potent antimicrobial components. Although inflammatory responses are crucial for host-survival (1), high concentrations of cytotoxic molecules lead to damage in surrounding tissues, which perpetrates further injury (1).

In an exposed organism, the initial innate immune response defines the outcome of the adaptive immune response. The adaptive immune response is designed to fine-tune and increase the efficacy of inflammation in clearing pathogens, speeding up resolution of infection or injury, and promoting wound healing. The adaptive and innate immune responses are guided by the expression profile of proteins that sense the environment and provide the necessary information to various immune cell subsets to orchestrate fast and efficient return to homeostasis. These proteins recognize specific molecular patterns and, thus, were named pattern recognition receptors (PRRs) (2, 3).

Molecular patterns that are recognized by the PRRs are broadly categorized into two groups: (1) those that accompany pathogens/microbes are called pathogen-associated molecular patterns (PAMPs) or microbial-associated molecular patterns and (2) those that are released by the injured tissues of dying cells called danger-associated molecular patterns (DAMPs). As of now, several families of PRRs were identified, including toll-like receptors (TLRs), C-type lectin receptors (CLRs), RIG-I-like receptors (RLRs), and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) (2). These PRRs play important roles in regulation of tissue inflammation.

Toll-like receptors are transmembrane proteins that are expressed in most cell types, either at the cell surface (TLR1, 2, 4, 5, 6, 10) or in endosomes (TLR3, 7, 8, 9). They can detect a variety of molecules, including proteins, lipopeptides, and nucleic acids (single-stranded RNA, double-stranded RNA, or CpG DNA). Ligand detection by TLRs initiates intracellular signaling cascades that activate inflammatory mediators, such as interferon regulatory factor (IRF) family members or NF-κB (4). CLRs are another family of PRRs that bind to carbohydrate structures, including mannose, fucose, and glucan on pathogens. They are mainly expressed on the surface of antigen-presenting cells (APCs), such as monocytes, macrophages, and dendritic cells (DCs) (5). The binding of pathogens to CLRs leads to its internalization, degradation, NF-κB activation, and subsequent antigen presentation to the T cells. Alternatively, RLRs are cytosolic PRRs that are expressed by both immune and non-immune cells that sense cytoplasmic RNA. During a viral infection, RLRs recognize viral dsRNA in the cytoplasm and activate antiviral signaling pathways, including Type I interferon and NF-κB. There are three members in RLRs family: RIG-I, melanoma differentiation-associated gene 5 (MDA5), and laboratory of genetics and physiology 2 (6).

Nucleotide-binding oligomerization domain-like receptors are the most recently discovered group of PRRs (7, 8). They were first described in plants, where they were shown to provide protective immunity against infection. As a protection mechanism, plants employ PRRs, such as intracellular immune receptors termed nucleotide-binding site leucine-rich repeat (LRR) proteins, which are structurally similar to mammalian NLRs. The importance of NLRs in regulating inflammation is highlighted by their evolutionary conservation across vertebrate species and the association of genetic mutations in several NLR genes with autoinflammatory diseases (9). NLRs were previously grouped under the term CATERPILLAR [Caspase-recruitment domain (CARD) transcription enhancer, R (purine)-binding, pyrin, lots of leucine repeats] gene family (10). Other research groups have named these proteins NOD-LRR family and NACHT [domain present in NAIP, class II transactivator (CIITA), HET-E, and TP1]-LRR family (8, 10). The study of NLR gene family emerged in the early 2000s following the discovery of their structural similarity to the CIITA, which is the master regulator of MHC class II transcription (11). NLR genes quickly surfaced as important mediators in apoptosis, immune responses, and inflammatory diseases. Currently, NLRs include 23 members in humans and at least 34 members in mice (12).

Structurally, NLRs consist of three highly conserved domains with the C-terminal region leucine-rich repeat (LRR), which is thought to be responsible for ligand binding; the central nucleotide binding ATPase domain NACHT/NBD (also known as NOD), which promotes oligomerization and activation; and the N-terminal domain, which contains either a CARD or pyrin domain (PYD) and is responsible for protein–protein interaction (13) (**Figure 1**). NLRX1 is an exception to this rule, instead of expressing an N-terminal protein-protein interaction domain, it possesses mitochondria-localization sequence (14). NLRs can be categorized by their structure and by their function. By the structure of the N-terminal domain, members of the NLR family are categorized into at least four subfamilies, including (1) NLRAs are characterized by the expression of acidic transactivation domain, (2) NLRBs contain baculovirus inhibitor of apoptosis protein repeat (BIR), (3) NLRCs possess CARD or an undefined domain, and (4) NLRPs contain PYD (**Figure 1**) (15–17).

Based on their function, NLRs can be classified into two main categories, non-inflammasome and inflammasome forming as depicted in **Figure 2**. Non-inflammasome NLRs can be further categorized into NF-κB regulators and transcription factors. Some NLRs, such as NLRP12, have been reported to play anti-inflammatory and proinflammatory roles depending on the experimental condition or the type of stimuli. Additionally, some NLRs act as transcription factors, such as CIITA and NLRC5, that indirectly regulate the immune response by tuning the expression of MHC II and I on APCs (18).

In this review, we provide an overview of the role of NLRs in inflammation during MS. The main focus of the review is on the innate immune response with the special emphasis on negative regulators of inflammation. Although the majority of research is devoted to the stimulators of inflammation, in our opinion, the endogenous inhibitors of inflammation have

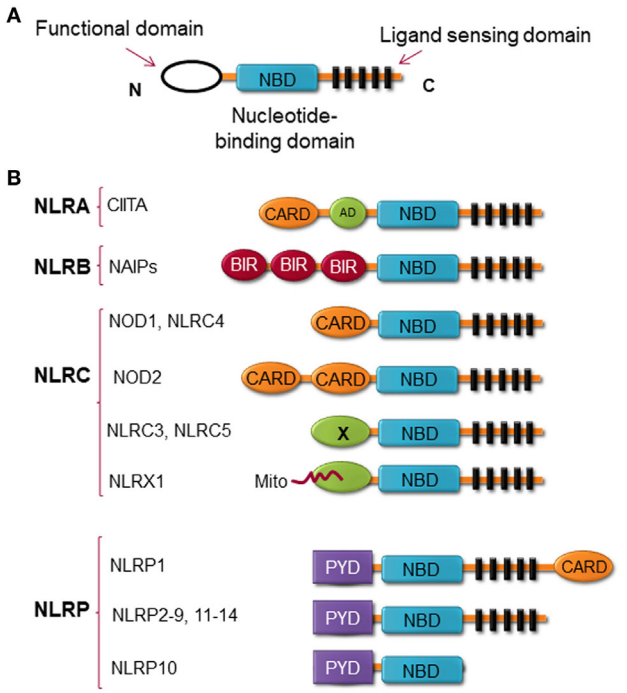

FIGURE 1 | Nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) structure. **(A)** The general structure of NLRs, consist of three domains, including functional domain, nucleotide binding and oligomerization domain, and ligand sensing domain. **(B)** Classification of NLRs based on the nature of their functional domain: NLRA, an acidic transactivation (AD) domain; NLRB, a baculovirus inhibitor of apoptosis protein (IAP) repeat (BIR); NLRC, a caspase-recruitment and activation domain (CARD); and NLRP, a pyrin domain (PYD). In NLRC subfamily, the X displays an unknown domain that has no homology with the other NLR members. Mito is the mitochondria-localization sequence that directs NLRX1 to the mitochondria.

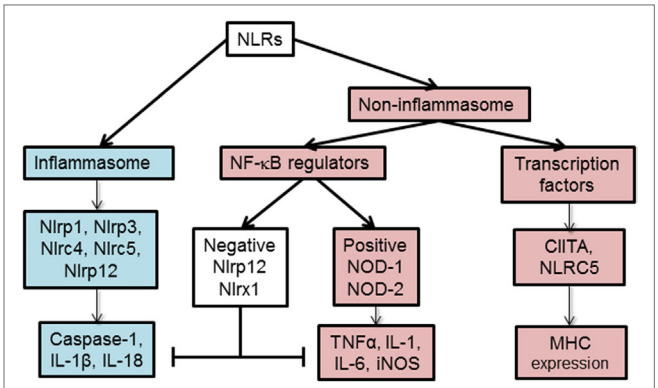

FIGURE 2 | Functional characterization of nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs). NLRs can be classified depending on their mechanism of action to inflammasome and non-inflammasome forming NLRs. The inflammasome forming NLRs assemble inflammasome that activates caspase-1 and promotes the production of inflammatory cytokines, IL-1β and IL-18. In the group of non-inflammasome forming NLRs, some NLRs regulate MHC II expression, while other NLRs regulate NF-κB signaling. The regulators of NF-κB consist of NLRs that enhance (NOD-1, NOD-2) or inhibit (NLRP12, NLRX1) NF-κB signaling pathway. The negative NLRs, NLRP12 and NLRX1, can inhibit both inflammasome-dependent and -independent cytokine production. NLRC5 and NLRP12 have been described to influence both inflammasome and non-inflammasome signaling pathways in a cell- and stimuli-dependent fashion.

a promising future as therapeutic targets for inflammatory disorders.

MULTIPLE SCLEROSIS

Multiple sclerosis is a devastating pathology that is diagnosed in young people predominantly between the ages of 20–40. Among neurological diseases, MS is the most common cause of disability in young adults (19). It is accompanied by the progressive decline of neurological functions, including vision and cognitive impairments and deterioration of sensory and motor functions (20).

Multiple sclerosis is a lymphocyte-mediated autoimmune disease (19). Indeed, ablation of adaptive immunity or inhibition of lymphocyte migration has been shown to slow down or even reverse the course of MS. However, such extreme therapies are associated with increased risk of fatal infections due to immunosuppression (21). Interestingly, all people possess autoreactive lymphocytes that patrol the central nervous system (CNS) during bacterial and viral infections, but only a relatively small number of them develop MS. This suggests the existence of predisposing factors other than lymphocytes-associated factors in MS patients.

Epidemiological studies suggest that MS results from the contribution of both genetic and environmental factors (22). Interracial studies and studies with monozygotic twins strongly suggest a genetic component of the disease, while geographical and migratory studies point to the involvement of environment in the MS pathology (23). The point of consensus between environmental and genetic theories of the etiology of MS is that in all cases immune system is deregulated. However, what remains uncertain is which components of the immune system and inflammatory response are the contributors and which are the result of the disease process. A distinguishing feature of MS pathology from other inflammatory diseases are the MS plaques, also known as lesions, which are widely spread throughout the CNS, particularly in the periventricular white matter, optic nerve, brain stem, and spinal cord areas. Pathological features of these plaques, include oligodendrocyte cell death, myelin destruction, axonal damage, glial scar formation, disruption and leakage of the blood–brain barrier (BBB), and the presence of inflammatory infiltrates composed of autoreactive T lymphocytes, microglia, macrophages, astrocytes, B lymphocytes, and ependymal cells (24–26). Activated macrophages, microglia, and astrocytes have been described in demyelinating lesions and are believed to play key roles in perpetuating disease progression in later stages of the disease (27–29). Most of the pathophysiological features of MS are reproduced in an rodent model called experimental autoimmune encephalomyelitis (EAE). It is based on immunization of animals with CNS antigens, including immunodominant peptides such as MOG35-55 and PLP (30). Although most of the pathophysiological changes were well characterized, behavioral description of EAE remains underdeveloped and includes

clinical scores that quantify the degree of ascending paralysis (31). Despite being widely criticized as being different from human MS, EAE model helped in developing multiple disease modifying drugs (30).

Innate Immune Response

Several studies from different laboratories suggest that CNS immune cells are activated before the appearance of clinical symptoms of MS and before T cell infiltration. For example, work from Dr. Fabry's group demonstrated activation of microglia and CNS DCs ahead of the infiltration of MOG-specific T cells in the olfactory bulb (32, 33), cerebellum, and along the white matter tracts (34). The activation of these cells was specific to EAE and was significantly increased compared to healthy mice and CFA-injected controls. Interestingly, an increase in microglia and DCs facilitates the migration of lymphocytes within the brain, which suggests that activation of these cells potentiate the effect of pathogenic T cells. Dr. Pham's (35) group and others demonstrated that inflammatory cells may utilize the rostral migratory pathway that is used by stem cells that go to the olfactory bulb. These observations put emphasis on early inflammatory events that precede the T cell infiltration and appearance of symptoms, which indicates that the activation of innate immune response potentiates CNS inflammation and may play a role in development of aberrant T cell responses.

Experimental autoimmune encephalomyelitis studies demonstrate that astrocytic responses coincide with early axonal damage (36). Astrocyte mediated inflammation is associated with inflammatory responses that are characterized by robust proliferation and hypertrophy of astrocytes that is termed astrogliosis. Astrocytes maintain the integrity of the BBB, provide for the energy needs of neurons, and are responsible for rapid reuptake of glutamate (37). Similar to macrophages/microglia, astrocytes express molecular machinery, which enables them to regulate inflammation and adaptive immune responses within the CNS (38–40). Therefore, astrocytes have all the features to orchestrate both an inflammatory response within the CNS and to regulate the influx and activity of lymphocytes. Astrocytes have been shown to upregulate inducible nitric oxide synthase (iNOS) in response to the elevated levels of proinflammatory cytokines (41). Increased iNOS activity generates NO, which is associated with the production of cytotoxic nitrites and nitrates that impede astrocyte-dependent glutamate uptake resulting in CNS damage (41). Activated astrocytes release inflammatory cytokines, such as IL-6, IL-1β, and TNF-α, that affect the tight junctions of endothelial cells, which allows the passage of immune cells through the BBB. Moreover, activated astrocytes secrete chemokines, such as MCP-1, RANTES, IP-10, SDF-1, and IL-8, which recruit leukocytes, such as monocytes, neutrophils, DCs, and lymphocytes, from the periphery to the CNS parenchyma, which further contribute to the cytotoxicity of the micro-environment (42). Proinflammatory cytokines and chemokines also activate microglia, which are the CNS-resident immune cells (43).

Microglia constantly monitor CNS environment and orchestrate innate immune response within the CNS parenchyma (44). Microglia originate from embryonic yolk sac at a very early stage of development, seed the brain, and stay there into adulthood (45–47). The morphology of microglia differs from that of conventional macrophages due to the presence of highly motile projections. Activated microglia have increased ability of phagocytosis and antigen presentation within the CNS (48–50). For a long time, activated microglia were considered to be indistinguishable from activated macrophages. Recently this notion was challenged; and TMEM119 emerged as a microglial marker (51). In many CNS pathologies, including MS, the number of microglia often increases in a phenomenon that is called reactive microgliosis (52, 53). Microglia release inflammatory mediators, such as iNOS, TNF-α, IL-1β, IL-6, IL-12, which aid in the recruitment of adaptive immune cells into the CNS (54).

Dendritic cells are professional APCs that uptake the antigen and travel to the local lymph node. Unlike other organs in the body, such as liver, skin, or intestine, the CNS parenchyma has a low number of DCs in the steady state (55). However, a recent work using the developmental and functional criteria demonstrated that DCs develop from their precursors (pre-DCs) in the meninges and choroid plexus of mice (56). In the case of neuroinflammation due to injury or infection, the BBB gets compromised and peripheral DCs infiltrate the CNS (55), where they contribute to antigen presentation and reactivation of encephalitogenic T cells (34, 57). Several studies also demonstrated the accumulation of DCs in white matter lesions and cerebrospinal fluid (CSF) of MS patients (58, 59).

Adaptive Immune Response

The role of T lymphocytes in MS pathogenesis has been well established (60, 61). After crossing the BBB, activated autoreactive T cells secrete inflammatory cytokines that activate macrophages and microglial cells. In turn, macrophages and microglia secrete chemokines that contribute to the recruitment of other T cells, DCs, and macrophages, which further amplifies the ensuing inflammatory cascade within the CNS. Furthermore, recruited T cells are activated by local APCs (62). Numerous $CD4^+$ T cell subsets have been implicated in MS, including T helper 1 (T_H1) and T helper 17 (T_H17) being the key components in the inflammatory response (63). T_H1 differentiation is favored in the presence of IL-12. Once T_H1 cells are activated, they release proinflammatory IFN-γ, TNF-α, GM-CSF, and IL-2 cytokines (62). T_H17 differentiation and development occur in the presence of IL-1, IL-23, IL-6, and TGF-β. Activation of this subtype of $CD4^+$ T helper cells results in secretion of IL-17A, IL-17F, IL-9, IL-21, IL-22, TNF-α, and GM-CSF (62, 64). Furthermore, $CD8^+$ T cells are also implicated in MS and are primarily found in the outer boundary of the lesions and in the perivascular area (62, 64). Interestingly, $CD4^+$ T cells were shown to play a role in the initial stages of lesion formation, whereas $CD8^+$ T cells were shown to be involved in the amplification of the inflammatory response, which resulted in damages (62, 63, 65). During inflammation, B cells and plasma cells are also recruited to the CNS. Plasma cells produce specific antibodies to myelin antigens that initiate the complement cascade, leading to destruction, opsonization, and subsequent phagocytosis of the myelin sheath (66).

Inflammatory Nature of MS

Despite extensive efforts to define MS immunopathology, the origin of the disease is still a matter of debate. The presence of autoreactive T and B cells in the CNS strongly supports the hypothesis that MS is primarily caused by an aberrant immune response against the CNS antigens, particularly myelin, in which chronic immune responses cause oligodendrocyte death and progressive demyelination (outside-in model of MS) (**Figure 3**) (67). It is still unknown how myelin-specific T cells are activated in the periphery. There are studies that support the activation of myelin-specific T cells by infectious agents (molecular mimicry) or non-specific T cells by superantigens (bystander activation) (68). Pathogens, such as *Mycoplasma pneumoniae* and *Chlamydia pneumoniae*, viruses, such as *Epstein Barr virus* and human *herpes virus*, and enterotoxins produced by *Staphylococcus aureus* are shown to be associated with the development or exacerbation of MS (69). The gut microbiome, which consists of digestive tract-associated microbes, actively regulate the homeostasis of the immune system. It has been suggested that dysbiosis may lead to dysregulation of the immune responses both in the periphery and the CNS (70).

Inside-out model of MS presents the idea that MS is primarily initiated by a neurodegenerative event (**Figure 3**). In this model, the oligodendrocyte injury or death would be the trigger of the CNS inflammation that presumably begins in the absence of a direct immune attack. Oligodendrocytes are extremely vulnerable to the oxidative stress due to their high metabolic rate, large intracellular iron stores, and low levels of antioxidative enzymes. Exposure to stress reactions or metabolic disturbances can lead to caspase activation and subsequent oligodendrocyte death (71). Oxidative stress also results in mitochondrial dysfunction, which causes axonal damage and oligodendrocyte apoptosis. As a result, myelin antigens are released into the peripheral circulation and activate autoreactive T and B cells that migrate to the CNS and induce inflammatory cascade.

Regardless of the nature of the primary trigger, both innate and adaptive immune responses are involved in potentiating demyelinating neuroinflammatory disease in MS (**Figure 4**). Although the infiltration of lymphocytes into the CNS is more prominent in the early stages of the disease, the disease becomes less dependent on lymphocytes and more neurodegenerative in later stages. Inflammation is present at all stages of the disease; it is triggered either by the infiltration of peripheral immune cells into the CNS or by the CNS-resident cells that respond to the CNS insult. From a classical point of view, NLRs are responsible for rapid sensing of PAMPs, such as products of microbial metabolism, and DAMPs, such as uric acid, ATP, nucleic acid, and ROS (72–75). The roles and functions of NLRs span beyond sensing of PAMPs and DAMPs; they are highly involved in the regulation of inflammatory pathways, such as NF-κB and mitogen-activated protein kinases (MAPK) (75–77). Next, we discuss positive and negative effect of NLRs on CNS inflammation (**Table 1**).

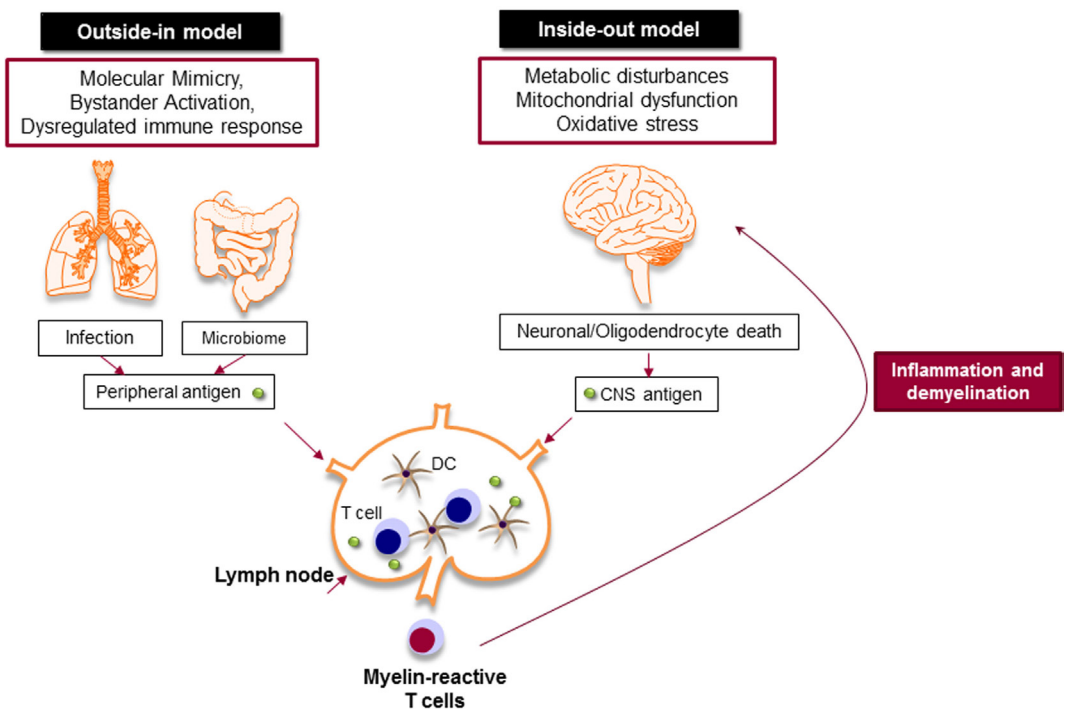

FIGURE 3 | Myelin-specific T cells are activated in the periphery by peripheral antigens or the central nervous system (CNS) antigens. In outside-in model, cross-activation of T cells by pathogen-derived molecules (molecular mimicry) or non-specific activation of T cells by superantigens (bystander activation) might be involved in the activation of myelin-specific T cells. The gut microbiome consists of digestive-tract associated microbes is also important to balance and regulate the immune response. The activated T cells attack CNS and cause inflammation and neurodegeneration. Inside-out model argues that the CNS inflammation primarily begins in the absence of a direct immune attack, in which neuronal/oligodendrocyte injury releases CNS antigen that triggers the immune response in the periphery.

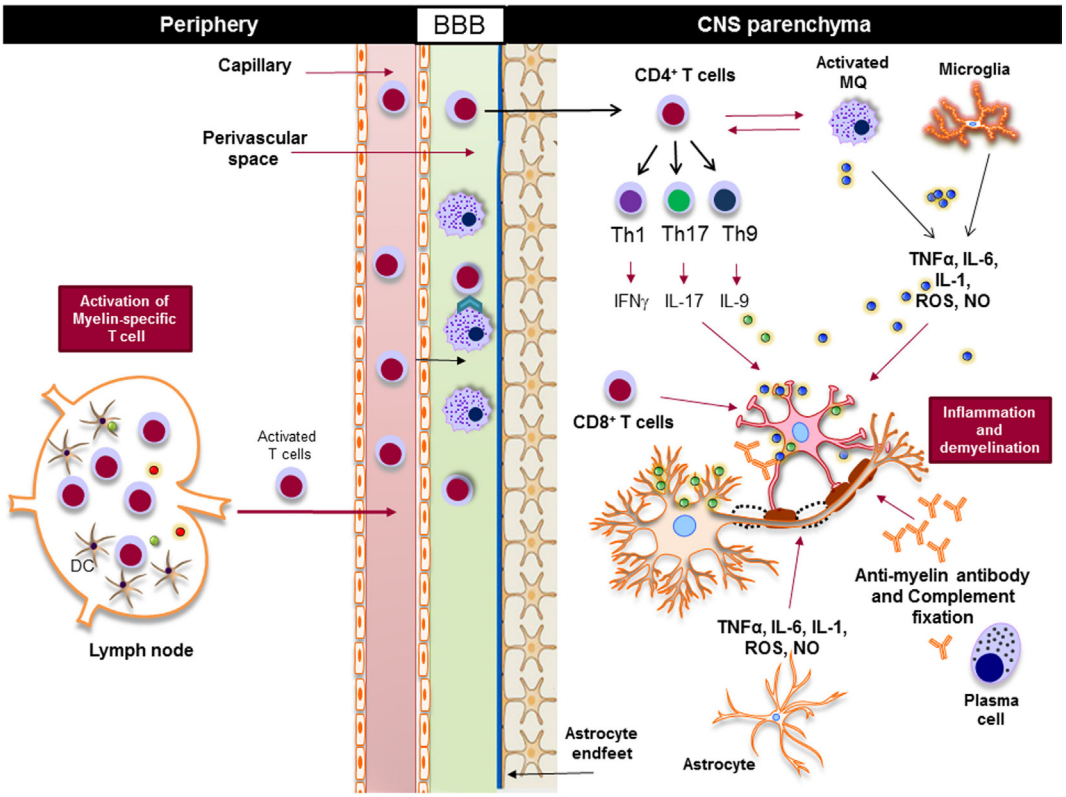

FIGURE 4 | Innate and adaptive immunity in the pathogenesis of multiple sclerosis. Myelin-reactive T cells are activated in the periphery and accumulate in the perivascular spaces, where they are reactivated by the central nervous system (CNS) myeloid cells, such as macrophages, and enter the CNS parenchyma. $CD4^+$ T cells are differentiated to different inflammatory subsets, such as Th1, Th17, and Th9, and once in the CNS they promote the activation of CNS-resident innate immune cells, such as macrophages and microglia. Inflammatory mediators, such as TNF-α, IL-6, IL-1, reactive oxygen species (ROS), and nitric oxide (NO), released from activated macrophages, microglia, and astrocytes damage oligodendrocytes and neurons, leading to demyelination. Activated cytotoxic $CD8^+$ T cells directly induce apoptosis in oligodendrocytes *via* FAS/FASL interaction, while plasma cells produce antimyelin antibodies that activate the complement system and damage oligodendrocytes.

NLRs AS POSITIVE REGULATORS OF INFLAMMATION

The activation of proinflammatory NLRs, such as NLRP1, NLRP3, NLRP6, NLRP7, NLRP12, NLRC4, and NAIP has been reported to result in the formation of inflammasome and production of potent inflammatory cytokines, such as IL-1β and IL-18 (78, 79). The assembly of inflammasome consists of binding of a regulatory NLR to ASC-adaptor molecule and an inactivated form of caspase-1. The formation of inflammasome activates caspase-1, which cleaves pro-IL-1β and pro-IL-18. The cleavage of IL-1β and IL-18 is necessary for their secretion (80). Activation of IL-1β is an essential innate immune cytokine, which is released primarily by myeloid cells in the CNS. It is involved in the leukocyte infiltration primarily by inducing the expression of cytokines, chemokines, and adhesion molecules (81). IL-18 is produced by a variety of cells, including monocytes, macrophages, microglia, and astrocytes. It plays a role in the recruitment of polymorphonuclear leukocytes by upregulating the expression of intracellular molecule-1 on endothelial cells (82). Activated caspase-1 has been shown to be significantly increased in MS patients and in EAE (83, 84). The role of inflammatory NLRs in the immunopathogenesis of MS is summarized in **Figure 5**.

NLRP1

NLRP1 was the first discovered inflammasome (85). It consists of NLRP1, ASC, the cysteine proteases caspase-1 (86). NLRP1 differs from other NLR proteins in that it has two signal transduction domains: a PYD and CARD (**Figure 1**) (87). NLRP1 is highly expressed by immune cells and is present at low levels in all tissues (88). In the CNS, expression of NLRP1 is highly dynamic and changes rapidly during various pathologies, such as trauma and stroke (86, 89). NLRP1 is expressed by neurons, microglia, and astrocytes and was shown to play a major role in neuronal death and CNS inflammation (90). There is strong evidence that suggests a link between NLPR1 and autoimmunity. Several studies found an association between NLRP1 and vitiligo (91), autoimmune thyroid diseases (92), and type I diabetes (93). In a recent publication, Maver et al. linked homozygous missense variant in NLRP1 gene (Gly587Ser) with familial forms of MS (94). Also, Bernales et al. found several *NLRP1* compound heterozygote

TABLE 1 | The role of inflammatory and anti-inflammatory nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) in multiple sclerosis (MS) patients and mouse MS models.

		Subjects	Major findings	Ref.
Inflammatory NLRs	NLRP1	MS patients	A homozygous missense variant in NLRP1 (Gly587Ser) was associated with familial MS	(94)
		MS patients	Compound heterozygous mutation was observed in several MS patients	(95)
	NLRP3	EAE	*Nlrp3*$^{-/-}$ mice developed ameliorated EAE, associated with a significant reduction of the inflammatory infiltrate to the CNS and lower production of IL-18, IFN-γ, and IL-17	(99)
		EAE	*Nlrp3*$^{-/-}$ mice were resistant to EAE with decreased inflammatory cell infiltration to the CNS. The activation of NLRP3 inflammasome in APCs is crucial for T cell migration to the CNS	(105)
		EAE	High-dose adjuvant induced severe EAE and neuronal damage in *Nlrp3*$^{-/-}$ mice, which was inflammasome-independent and resistant to IFN-β therapy	(108)
		MS patients	IFN-β treatment attenuated the course and severity of MS by reducing the activity of NLRP3 inflammasomes	(112)
		MS patients	Q705K polymorphism (rs35829419) results in overactive NLRP3 inflammasome, which was associated with IFN-β response in MS patients	(112)
	NLRC4	Cuprizone mouse model	NLRC4 inflammasome in microglia and astrocytes is associated with neuroinflammation and demyelination	(115)
	NOD1 and NOD2	EAE	*Nod1*$^{-/-}$ and *Nod2*$^{-/-}$ mice were highly resistant to EAE. Reduced number of activated APC and activation of T cells in the CNS were observed	(120)
Anti-inflammatory NLRs	NLRP12	EAE	*Nlrp12*$^{-/-}$ mice developed EAE earlier with more severe clinical and pathological outcomes. The absence of Nlrp12 results in an increased inflammatory response in microglia	(126)
		EAE	*Nlrp12*$^{-/-}$ mice had ameliorated EAE course with atypical symptoms, including ataxia and impaired balance control, which was associated with increased production of IL-4	(128)
	NLRX1	EAE	Protective role of NLRX1 in EAE. *Nlrx1*$^{-/-}$ mice showed increased macrophage/microglial activation and cytokine production, which resulted in increased tissue damage	(155)

mutations in MS patients (95). The association of NLRP1 and the pathophysiology of MS needs further investigation.

NLRP3

Given its prominent role in a number of diverse diseases, NLRP3 is by far the most well-known activator of inflammasome signaling. Mutations in NLRP3 gene lead to several autoinflammatory disorders referred to as the cryopyrin-associated periodic syndromes (96). NLRP3 is activated in two steps. The first step is priming the cells by PAMPs or DAMPs *via* TLRs, which leads to the activation of NF-κB signaling that triggers the expression of inflammasome-related components, including NLRP3, pro-IL-1β, and pro-IL-18. The second step is oligomerization of NLRP3 and its association with an adaptor protein ASC and pro-caspase-1. This complex triggers the activation of caspase-1 that cleaves pro-IL-1β and pro-IL-18 into their mature and secreted forms: IL-1β and IL-18 (97). Activation of the NLRP3 inflammasome also results in pyroptosis, a caspase 1-dependent cell death, which is a highly inflammatory. Pyroptosis results in cell lysis and the release of cytosolic components into the extracellular environment (98).

Previous studies demonstrated that NLRs and their adaptors could positively influence the development and the severity of EAE (99). Deletion of *Nlrp3*, *ASC*, or the *caspase-1* gene resulted in protection against EAE (99, 100). NLRP3 causes severe inflammatory symptoms in EAE by producing more IL-1β and IL-18, which stimulate the development and activation of T_H1/T_H17 cells and enhance their infiltration into the CNS (99). It was shown that NLRP3 inflammasome assembles in human $CD4^+$ T cells and initiates caspase-1 activation and IL-1β production, which results in promoting IFN-γ secretion and T_H1 differentiation in an autocrine manner (101). Other studies demonstrate key roles for the inflammasome-mediated IL-1 production in the induction of GM-CSF by both T_H1 and T_H17 cells in EAE (102). In an alternative pathway, NLRP3 inflammasome engages caspase-8 instead of caspase-1 (103). The importance of NLRP3-caspase-8 inflammasome was recently shown in the production of IL-1β by T cells that support the survival of T_H17 cells in EAE (104). Moreover, NLRP3 inflammasome in APCs played a critical role in upregulating chemotactic proteins, such as osteopontin, CCR2, and CXC chemokine receptor (CXCR) 6, in T_H1 and T_H17 cells, thereby inducing T cell migration to the CNS in EAE (105).

Demyelinating neuroinflammatory disease was shown to develop in the absence of NLRP3 inflammasome, which resulted in more severe EAE (100, 106). These studies showed that the induction of potent innate immune responses with high dosages of heat-killed mycobacteria (Mtb) in adjuvants drives aggressive neuronal damage and EAE disease in mice that are deficient in either ASC or NLRP3 (107). Disease progression in this more aggressive model of EAE (referred to as type B EAE) was found to be dependent on membrane-bound lymphotoxin-β receptor (LTβR) and CXCR2 (108).

Many reports demonstrate detrimental role of NLRP3 inflammasome in MS patients. The expression of caspase-1 and IL-18 are elevated in peripheral mononuclear cells from MS patients compared to those from healthy controls (83). Moreover, the levels of IL-1β are upregulated in CSF of MS

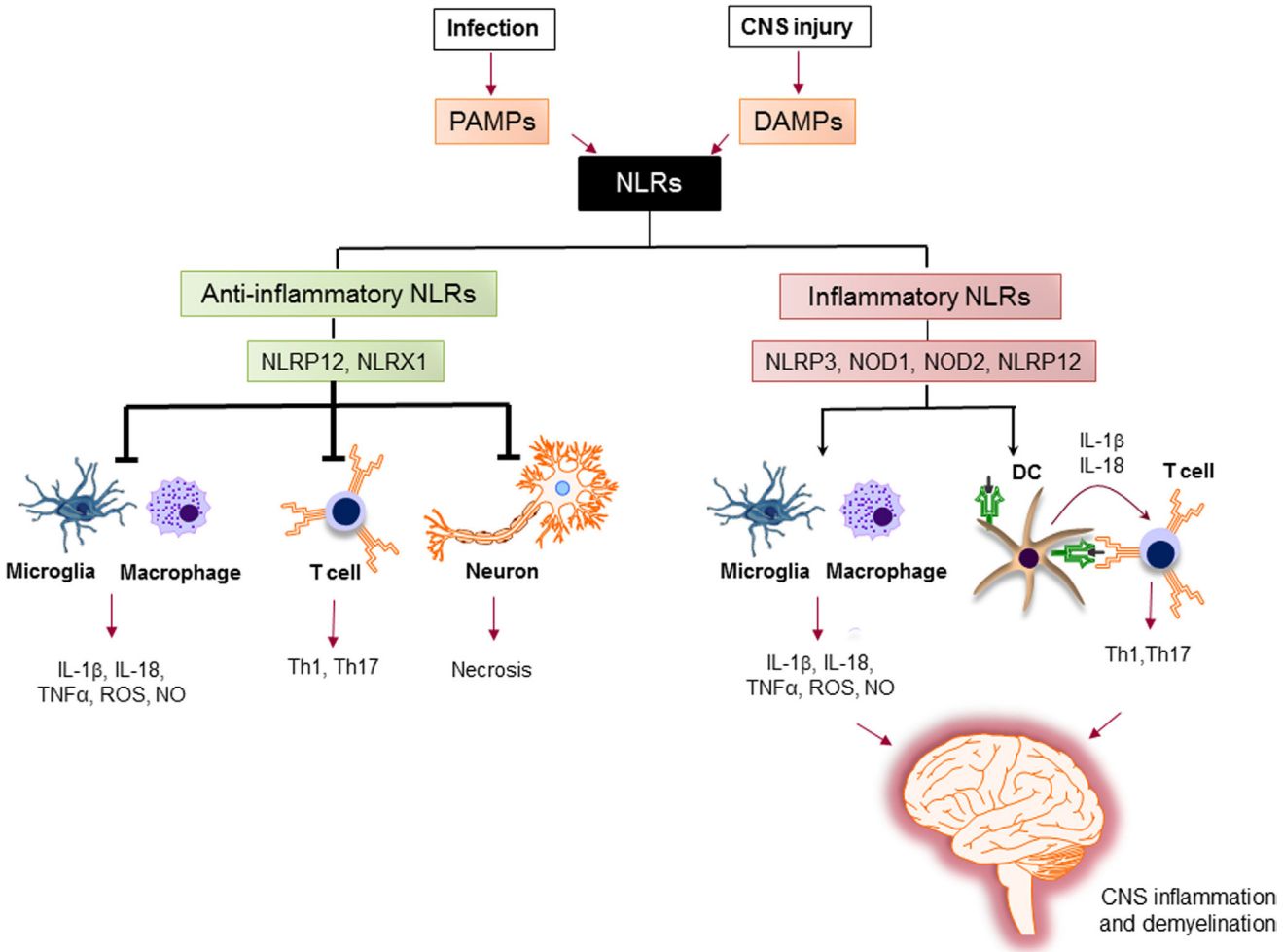

FIGURE 5 | The role of nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) in regulation of inflammation in multiple sclerosis (MS). Pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs) trigger the immune response *via* NLRs. Upon sensing ligands, inflammatory NLRs not only initiate inflammatory response in innate immune cells, such as macrophages and microglia, but also bridge the immune response from innate to adaptive immune response *via* instructing T cell response by dendritic cells (DCs) to generate different subsets of pathogenic T helper subsets (e.g., Th1, Th17). On the other hand, anti-inflammatory NLRs inhibit the production of inflammatory mediators by macrophages and microglia, suppress the differentiation of T cells to inflammatory subsets, and protect neurons from necrosis. Thereby, the increased activation of inflammatory NLRs and the impaired function of anti-inflammatory NLRs lead to central nervous system inflammation and demyelination in MS.

patients and correlate with the progression of MS (109). MS treatments, such as glatiramer acetate and IFN-β, elevate the levels of endogenous IL-1 receptor antagonist in MS patients (110, 111). A study by Malholtra et al. showed that the IFN-β treatment attenuated the course and severity of MS by reducing the activity of NLRP3 inflammasomes *via* suppressing caspase-1 dependent IL-1β secretion (112). The Q705K polymorphism (rs35829419) in exon 3 produced an overactive NLRP3 inflammasome, which was associated with IFN-β response in MS patients (112).

Beyond its role in forming inflammasome, NLRP3 is a transcriptional regulator of T_H2 differentiation in a T cell-intrinsic manner. A recent study by Bruchard et al. showed that NLRP3 acts as a key transcription factor in T_H2 differentiation in conjunction with IRF4. NLRP3 binds and activates *IL-4* promoter in T_H2 cells in an inflammasome-independent manner (113). The T cell-intrinsic role of NLRP3 needs further investigation.

NLRC4

NLRC4 is well characterized in bacterial infection, such as *Salmonella typhimurium* and *Legionella pneumophila* (114). In sterile inflammation, the CNS-associated DAMP, lysophosphatidylcholine, activates NLRC4 inflammasome in microglia and astrocytes. A recently published study revealed that the activation of NLRC4 inflammasome in microglia and astrocytes is associated with neuroinflammation and demyelination in cuprizone mouse model (115), a model of toxin-induced demyelination without the activated adaptive immunity (116). The increased NLRC4 expression in the lesions of human MS brains confirms the association between NLRC4 and neuroinflammation in MS (115).

Other Proinflammatory NLRs

Other proinflammatory NLRs, such as NOD1, NOD2 (117), and NLRP10 (118), induce inflammation, which is independent of inflammasome formation. These NLRs upregulate NF-κB and activate MAPK pathways (119). Furthermore, NOD2 interacts with mitochondrial antiviral signaling protein (MAVS) (77), which is essential for the production of IFN-β to suppress virus replication during viral infections. Shaw et al. showed that *Nod1*$^{-/-}$ and *Nod2*$^{-/-}$ mice are highly resistant to EAE due to the reduced number of activated APC, which leads to a reduced activation and expansion of T cells in the CNS (120). These findings collectively demonstrate that NLR proteins can exacerbate MS, either *via* formation of inflammasome or stimulation of inflammatory pathways, such as NF-κB and MAPK.

NLRs AS NEGATIVE REGULATORS OF INFLAMMATION

The activation of PRR by PAMPs and DAMPs is negatively regulated by members of NLRs family, including NLRP12, NLRX1, and NLRC3. The role of anti-inflammatory NLRs in regulation of inflammation in MS is summarized in **Figure 5**.

NLRP12

NLRP12 is a pyrin-containing NLR protein that is expressed in cells of myeloid origin and is formerly known as RNO, PYPAF7, and Monarch-1 (121, 122). The HUGO Gene Nomenclature Committee approved the name of NLRP12 for this gene. Two research groups simultaneously cloned the full-length sequence of human NLRP12 (121, 122) and, later, it was identified in HL60 human leukemic cell line (123). In humans, NLRP12 is expressed in neutrophils, eosinophils, macrophages, monocytes, and immature DCs (123–125).

Since the discovery of NLRP12, there have been contrasting reports that demonstrated both proinflammatory and anti-inflammatory roles of NLRP12 in cell-type and stimuli-specific manners (126–130). Early studies showed that NLRP12 is an inflammatory NLR that interacts with ASC to form inflammasome, leading to caspase-1 activation and release of mature IL-1β. Evidence for the involvement of NLRP12 in inflammasome formation and activation are largely derived from *in vitro* studies that used overexpression systems (121). Recent studies showed the role of NLRP12 in activation of inflammasome by intracellular pathogens, such as *Yersinia Pestis* and *Plasmodium* infection (130, 131), but not by other pathogens, such as *Salmonella, Klebsiella, Escherichia, Mycobacterium*, and *Listeria* species (127, 132, 133). Taken together, these studies established a biologically relevant role for the NLRP12 inflammasome in innate immune responses against pathogens; however, the exact molecule that triggers NLRP12 inflammasome remains unknown.

Alternatively, there are studies that identified NLRP12 as a negative regulator of inflammation that inhibits NF-κB signaling in innate immune cells. It was shown that the activation of human peripheral blood granulocytes and monocytes by TLR4 or TLR2 agonists (*E. coli* LPS or synthetic lipopeptide Pam3Cys, respectively) reduces the expression of NLRP12 (122). Moreover, the expression of NLRP12 declines significantly in myeloid cells (THP-1 human monocytic cell line) after *in vitro* stimulation with live bacteria, such as *Mycobacterium* or *Plasmodium,* or cytokines, such as TNF-α or IFN-γ (134). When *NLRP12* was knocked down in THP1 cells using shRNA, the expression levels of proinflammatory cytokines significantly increased following LPS or *M. tuberculosis* treatment (134). A transcriptional repressor called B lymphocyte-induced maturation protein-1 is induced by TLR stimulation and downregulates NLRP12 expression by binding to *Nlrp12* promoter and recruiting histone deacetylases (135, 136).

Mechanistically, NLRP12 was shown to suppress both canonical and non-canonical NF-κB signaling pathways. The canonical pathway is mediated by translocation of the NF-κB RelA/p50 subunits to the nucleus and is activated in response to TNFR, IL-1R, or TLR signaling. NLRP12 inhibits hyperphosphorylation of the receptor-associated kinase (IRAK-1) that triggers IκBα degradation and p50 nuclear translocation (134, 137). The non-canonical NF-κB pathway is triggered by signaling through receptors, such as CD40, LTβR, or BAFF-R. The signal activates NF-κB inducing kinase (NIK) and IKKα, which leads to p100 cleavage and nuclear translocation of p52 dimers. In the non-canonical NF-κB signaling pathway, NLRP12 interacts with TNF receptor-associated factor (TRAF3) and NIK, which leads to the degradation of NIK and subsequent reduction of p100 cleavage to p52 (**Figure 6**) (125, 138). ATP binding to NLRP12 is crucial for its inhibitory function, as the cells with an NBD mutant form of NLRP12 are not able to inhibit NF-κB activation. As a result, they produce high levels of proinflammatory cytokines and chemokines (139).

Apart from its inhibitory role in monocytes/macrophages, NLRP12 was shown to enhance the migration of DCs to the draining lymph nodes (140). In the absence of NLRP12, the migration of DCs to the local lymph nodes is significantly decreased, while their abilities for antigen presentation, inflammasome activation, or production of inflammatory cytokines are not impaired. Therefore, *Nlrp12*$^{-/-}$ mice fail to generate robust hypersensitivity response against the topical application of hapten-like oxazolone (140).

Consistent with the regulatory function for NLRP12 in innate immune cells, *Nlrp12*$^{-/-}$ mice were shown to be highly susceptible to inflammatory diseases of intestine, such as experimental colitis and colon cancer (137, 138). This is due to the increased activation of NF-κB in macrophages of *Nlrp12*$^{-/-}$ mice, which results in the production of proinflammatory cytokines and mediators. Consistent with these findings, our work demonstrated a protective role of NLRP12 during CNS inflammation. We showed that lack of NLRP12 potentiated the course of EAE (126). Indeed, *Nlrp12*$^{-/-}$ mice developed earlier and more severe EAE compared to the WT mice (126). *In vitro* experiments also confirmed the inhibitory role of NLRP12 in microglia activation and the production of proinflammatory mediators, such as iNOS expression, NO, TNF-α, and IL-6 (126).

Alternatively, a study by Lukens et al. proposed that NLRP12 provokes CNS inflammation in EAE that is related to its regulatory function in T cells (128). Early reports mainly described NLRP12 expression and its anti-inflammatory function in innate

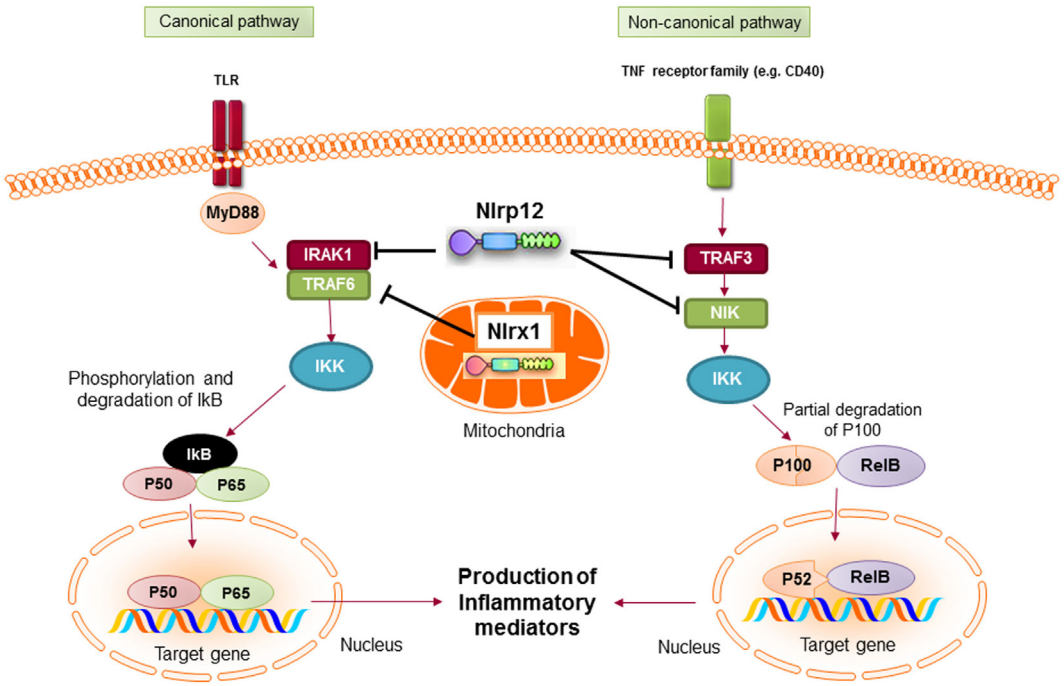

FIGURE 6 | Inhibitory nucleotide-binding oligomerization domain (NOD)-like receptors put the brake on NF-κB activation *via* canonical and non-canonical pathways. Both NLRX1 and NLRP12 inhibit the activation of NF-κB canonical pathway following toll-like receptor (TLR) stimulation. Nlrx1 interacts and inhibits TNF receptor-associated factor (TRAF) 6, while NLRP12 inhibits the phosphorylation of IRAK-1. NLRP12 can also inhibit non-canonical NF-κB signaling through regulation of TRAF3 and NF-κB inducing kinase (NIK).

immune cells with myeloid origin, such as DC and macrophages (137, 140). Recently, Lukens et al. reported a T cell-intrinsic role for NLRP12, which negatively regulates NF-κB signaling, T cells proliferation, and the secretion of $T_H1/T_H2/T_H17$ cytokines (128). Therefore, *Nlrp12*$^{-/-}$ T cells developed enhanced inflammatory symptoms in T-cell-mediated autoimmune diseases such as colitis and atopic dermatitis (138, 140). These findings support our observation that lack of NLRP12 was associated with increased neuroinflammation and severe scores of EAE (126). Lukens' study shows that the absence of NLRP12 promoted IL-4 secretion resulting in the development of atypical EAE disease symptoms, including ataxia and impaired balance control (128).

NLRP12 also plays a positive role in T_H1/T_H17 differentiation. Using an *in vitro* T-cell differentiation assay, Cai et al. reported a reduced T_H1 and T_H17 differentiation in *Nlrp12*$^{-/-}$ T cells, while Th2 differentiation remained similar to WT T cells (141). In response to *Brucella abortus in vivo*, Silveira et al. showed that *Nlrp12*$^{-/-}$ T cells produced more IFN-γ compared to WT controls. The study did not investigate whether the increased production of IFN-γ was due to high numbers of T_H1 cells; however, they demonstrated that NLRP12 negatively regulated IL-12 production by macrophages following *B. abortus* infection, which skewed T cell differentiation to T_H1 (129). Further research is needed to understand how NLRP12 regulates T cell activation and differentiation.

Regarding the dual role of NLRP12 in the regulation of inflammation (142), the inconsistency of results across laboratories might be related to the different environmental conditions that result in different microbiomes, and different knockout strategies to delete NLRP12 gene, which may produce uncontrolled variable phenotypes. Interestingly, two recent studies may provide clues to explain some of the inconsistencies in the NLRP12 literature. In one study, it was shown that some C57BL/6 colonies have acquired a missense mutation in the *Nlrp12* gene and that this can affect neutrophil responses (143). In the second study, genetic ablation of NLRP12 was found to cause significant changes in microbiota landscape in mice (144). Interestingly, they found that cohousing *Nlrp12*$^{-/-}$ mice with WT mice attenuates intestinal inflammatory disease in NLRP12-deficient mice. Collectively, these two studies demonstrate that it is important when evaluating a role for NLRP12 in disease to take into consideration differences in microbiota composition in *Nlrp12*$^{-/-}$ mice colonies and choice of C57BL/6 wild-type controls. Going forward it will be particularly interesting to determine how modulation of intestinal microflora landscape in NLRP12-deficient mice influences inflammatory responses and disease progression in other models of disease.

NLRX1

NLRX1 is a recently characterized member of the NLR family that is uniquely localized in the mitochondria (145). The protein is expressed widely in all tissues with the highest expression in heart and muscle (146). Initial studies showed that NLRX1 was located in the outer membrane of mitochondria (145). However, later studies demonstrated that NLRX1 is predominantly

located in the matrix of mitochondria (14, 147). The localization of NLRX1 in mitochondria is due to the presence of a functional N-terminal mitochondrial-localization sequence (14). In mitochondria, NLRX1 interacts with UQCRC2, a part of the ubiquinol-cytochrome c reductase complex, that is a part of the mitochondrial respiratory chain (MRC). The MRC generates an electrochemical signal that drives ATP production (148). It also produces ROS in eukaryotic cells that could cause oxidative stress and tissue damage (149). A study by Tattoli et al. showed that NLRX1 induces ROS production in cells treated with TNF-α and double-stranded RNA, which results in increased activation of inflammatory pathways, such as NF-κB (146). Alternatively, Xia et al. reported that NLRX1 acts as a negative regulator of TLR-mediated NF-κB signaling. In resting cells, NLRX1 interacts with TRAF6. However, after cell stimulation with LPS, NLRX1 rapidly dissociates from TRAF6 and binds to the IKK complex, leading to inhibition of IKKα/β phosphorylation and NF-κB activation (**Figure 6**) (150). Therefore, depending on the experimental conditions, NLRX1 can either activate or inhibit NF-κB signaling.

In the context of viral infections, NLRX1 acts as a negative regulator of the antiviral signaling pathway (145, 151). Once cells are infected with a virus, viral PAMPs are detected by the cytoplasmic RLRs and MDA5, which activate MAVS signaling pathway, resulting in the activation of IRF3, NF-κB, and transcription of type-1 interferon (IFN-1) (145, 151). Moore et al. reported that interaction between NLRX1 and MAVS prevents RIG-I from binding to MAVS, which results in inhibition of NF-κB activation and IFN-1 production (145). Consistent with this finding, Allen et al. showed that embryonic fibroblasts from *Nlrx1*$^{-/-}$ mice had increased production of type 1 IFN after viral infection as compared to WT controls (151). In contrast, Rebsamen et al. show NLRX1 has no effect on antiviral response. They reported that NLRX1$^{-/-}$ embryonic fibroblasts had normal cytokine production in response to Sendai virus infection (152). This finding is in agreement with Soares et al. study, which showed that antiviral signaling pathway is intact in *Nlrx1*$^{-/-}$ mice during both *in vivo* and *ex vivo* viral infections (153). Apart from its controversial role in antiviral immune response, NLRX1 can induce autophagy that deletes the cytosolic viral RNA and consequently results in the inhibition of type 1 IFN production. Lei et al. proposed that NLRX1 forms a multimeric complex with the cytosolic autophagy-related (ATG) proteins and a mitochondrial matrix protein, mitochondrial Tu translation elongation factor (TUFM), which is known to initiate autophagic responses (154). For this reason, NLRX1 deficient cells enhance type I IFN production and are more efficient to restrict the replication of a variety of viruses compared to WT cells.

Studies summarized here highlight the regulatory role of NLRX1 in the immune responses against viruses. However, emerging studies have also identified key roles for NLRX1 in multiple autoinflammatory and autoimmune disease models. For instance, a recent work by Eitas et al. demonstrated a protective role of NLRX1 in EAE by suppressing macrophage/microglial activation (155). In this study, *Nlrx1*$^{-/-}$ mice showed increased cytokine production and enhanced tissue damage during EAE compared to the WT mice (155). This finding is consistent with a recent study by Allen et al., which also showed the anti-inflammatory function of NLRX1 in sterile CNS inflammation, such as traumatic brain injury (156). Mechanical trauma to the CNS results in the disruption of the cellular microenvironment leading to massive necrotic and apoptotic loss of neuronal and glia populations. *Nlrx1*$^{-/-}$ mice exhibited significantly larger brain lesions and increased motor deficits following brain injury. Their data indicates that NLRX1 attenuates NF-κB signaling and IL-6 production in microglia (157).

It is also reported that NLRX1 regulates mitochondrial dynamics and cell death. Recently, our research group showed that NLRX1 protects the neuronal-like cell line (N2A cells) from necrosis (158). We found an increased number of mitochondria in NLRX1-overexpressed N2A cells compared to control cells, which was associated with increased phosphorylation of DRP1 and mitochondrial fission. As a result, NLRX1 switched the cell death from necrosis toward apoptosis, which inhibits neurodegeneration by preventing the release of inflammatory mediators in the tissue environment and maintaining the tissue homeostasis (158). Consistent with our observations, a study by Girardin's group showed that NLRX1 accelerates intrinsic apoptotic pathway induced by prolonged cellular stress or glucose starvation (159).

Interestingly, recent studies suggest that in addition to playing prominent roles in the regulation of innate immune cells, NLRX1 can also affect adaptive immunity by inhibiting T cell proliferation and differentiation (160). In dextran sodium sulfate-induced colitis mouse model, lack of NLRX1 results in enhanced T_H1- and T_H17-related inflammatory cytokines, such as IFN-γ, TNF-α, and IL-17, and consequently increased the severity of the disease (160). *In vitro* experiments revealed that *Nlrx1*$^{-/-}$ T cells have a greater ability to proliferate and differentiate into T_H17 cells. The T-cell intrinsic role of NLRX1 was confirmed in adoptive-transfer model of colitis. The *Rag*$^{-/-}$ mice receiving *Nlrx1*$^{-/-}$ T cells experienced more severe clinical disease and increased numbers of T_H1 and T_H17 cells in spleen and colonic lamina propria (160).

NLRC3

NLRC3 is predominantly expressed in cells of the immune system, particularly in T cells (134). NLRC3 functions as a novel suppressor of T cell activation. It inhibits NF-κB, AP-1, and NFAT transcriptional activation in Jurkat T cells downstream of CD3/CD28 stimulation or treatment with PMA/ionomycin (134). Studies of *Nlrc3*$^{-/-}$ mice confirmed the inhibition of proinflammatory signaling, K63-linked ubiquitination of TRAF6, and nuclear translocation of NF-κB by NLRC3 (161). Interestingly, it was shown that *nlrc3-like* gene is required for microglia development in Zebrafish (162). In *nlrc3-like* mutants, primitive macrophages gain an inflammatory phenotype with increased proinflammatory cytokines that prevent their migration into the brain and subsequent differentiation into microglia. This study suggests that *nlrc3-like* serves as a critical regulator of microglia development in Zebrafish; however, future studies in vertebrate models are needed to fully elucidate roles for NLRC3 in neuroinflammatory diseases.

NLRs in Other Neuroinflammatory Diseases

The importance of NLR proteins can be further appreciated by their crucial role in inflammatory diseases where a simple mutation in these genes can result in pathology (163) Cryopyrin-Associated Periodic Syndromes (CAPS) are a group of autoinflammatory syndromes resulting from an autosomal dominant mutation in the *Nlrp3* gene (164). Variants of NLRP1 proteins have been shown to be associated with vitiligo, an autoimmune disease resulting in areas of skin hypopigmentation as a consequence of melanocytes damage (91). Moreover, mutations in the NBD of *Nod2* gene result in Blau syndrome, an autosomal dominant disorder that is characterized by skin rashes, arthritis, and granulomatous uveitis (165). The proinflammatory NLRP3 contributes to the pathology of a broad spectrum of neurological diseases, such as stroke (166), traumatic injury (167), and neurodegenerative diseases, including Alzheimer (168) and amyotrophic lateral sclerosis (ALS) (169).

THERAPEUTIC POTENTIAL OF TARGETING NLRs IN DISEASE

Inhibition of Inflammatory NLRs

Targeting NLR-mediated inflammasome activation is an attractive therapeutic approach that is actively being investigated to treat a multitude of autoinflammatory and autoimmune disorders. As described above in greater detail, multiple NLRs are known to coordinate inflammasome-mediated production of IL-1β and IL-18, as well as a caspase-1-dependent form of cell death known as pyroptosis. Therapeutic molecules targeting IL-1 are being used in the clinic for many years. Currently, there are three approved IL-1 blockers, including the IL-1 receptor antagonist, anakinra; a soluble decoy receptor, rilonacept; and a neutralizing monoclonal anti-IL-1β antibody, canakinumab (170). There are no approved treatments to block IL-18 in humans at this time, however, a recombinant human IL-18 binding protein (Tadekinig alfa) is currently in clinical trials (171). Despite their notable efficacy, anticytokine drugs are not able to inhibit other inflammasome-associated pathologies, such as caspase-1-mediated pyroptosis. Therefore, therapeutics that directly target inflammasome activation may offer greater efficacy over strategies that only target inflammasome-derived cytokines. In this respect, two caspase-1 inhibitors have been recently developed and tested in clinical trials. These are orally absorbed prodrugs: Pralnacasan (VX-740) and Belnacasan (VX-765), that selectively inhibit the activity of caspase-1 (172).

IFN-β is one of the most widely prescribed disease modifying therapies for relapsing-remitting MS. IFN-β exerts its anti-inflammatory effect through the suppression of NLRP1 and NLRP3 inflammasome and IL-1β production, as it was shown in mouse bone marrow-derived macrophages and blood monocytes isolated from IFN-β treated MS patients (173). The NLRP3 inflammasome is associated with the response to IFN-β in patients with MS (112), which indicates that IFN-β specifically inhibits NLRP3 inflammasome.

There are two small-molecule inhibitors of inflammasome that specifically inhibit NLRP3 inflammasome. MCC950 is a diarylsulfonylurea-containing compound that blocks NLRP3-induced ASC oligomerization in mouse and human macrophages (174). MCC950 acts specifically on the NLRP3 inflammasome and does not inhibit the activation of NLRP1, AIM2, or NLRC4 inflammasomes (174). It was shown that treatment of mice with MCC950 delayed the onset and reduced the severity of EAE (174). The ketone metabolite β-hydroxybutyrate (BHB) is another small-molecule inhibitor of inflammasome that specifically inhibits NLRP3-induced ASC oligomerization (175).

Another approach for inhibiting inflammasomes is using MicroRNAs, the single-stranded non-coding RNA molecules that bind to the 3-untranslated region of mRNAs to regulate gene expression (176). MicroRNA-223 binds to a conserved site in the 3 UTR of the NLRP3 transcript, suppressing the protein expression, thus, inhibiting NLRP3 inflammasome (177). The therapeutic application of MicroRNA-223 is currently under investigation in animal models (178). Despite the availability of miRNA therapeutics in human clinical trials, none of them are currently known to target inflammasome signaling (179).

Previous studies suggest that mitochondrial dysfunction plays a crucial role in the pathogenesis of neurodegenerative disorders, such as Parkinson's disease (PD), Alzheimer's disease (AD), and MS (180, 181). Mitochondrial dysfunction generates ROS, which triggers NLRP3 oligomerization and activates inflammasome (182). Therefore, the inhibition of mitochondrial ROS (mtROS) using mitochondria-targeted antioxidants is another approach to suppress inflammasome (183). A recent publication shows that MitoQ, a mitochondria-targeted antioxidant derived from ubiquinone, attenuates experimental mouse colitis by inhibiting the NLRP3 inflammasome and the production of inflammatory cytokines (184). The neuroprotective effects of MitoQ have been confirmed in EAE mice, in which treatment with MitoQ reduced axonal inflammation and neurological disabilities (185). These findings suggest that novel mitochondria-targeted antioxidants could be promising therapeutic targets for MS treatment (186).

Stimulation of Anti-inflammatory NLRs

NF-κB is a master regulator inflammation that plays pivotal roles in the transcriptional control of a vast majority of inflammatory mediators, including TNF-α, IL-6, pro-IL-1β, pro-IL-18, procaspase-1, and NLRP3 (187). In addition to coordinating the expression of many inflammasome-related molecules, NF-κB can also potently affect inflammatory responses through its regulation of chemokine and adhesion molecule production, and its control of cell proliferation and differentiation (188). Therefore, the activation of NF-κB pathway is not only required for the assembly of inflammasome complex but required for the activation and recruitment of inflammatory cells to the site of inflammation. The contribution of NF-κB in such a broad spectrum of inflammatory responses has spurred great interest in the development of NF-κB inhibitors to treat MS.

Multiple studies have demonstrated that NF-κB inhibition, both in peripheral immune cells and in the CNS, is protective in

EAE, suggesting that pharmacological targeting of the NF-κB pathway might have a therapeutic effect in MS. A number of currently prescribed MS drugs, including fingolimod, teriflunomide, and dimethyl fumarate, have been reported to indirectly modulate NF-κB signaling (189). There are several NF-κB specific inhibitors, such as DHMEQ and bindarit, which prevent the nuclear translocation of the p65 (190) or reduce the phosphorylation of IκBα and p65 (191). NF-κB specific inhibitors showed potent anti-inflammatory and anticancer activities in many animal models (192). However, their anti-inflammatory activity in autoimmune diseases requires further investigation.

During the proinflammatory response, anti-inflammatory NLRs provide simultaneous and opposing down-regulation of inflammation that target not only immune cells and their mediators but also CNS-resident cells. Therefore, targeted approaches to boost the expression or function of anti-inflammatory NLRs would serve as a novel strategy to treat neuroinflammatory disease. Anti-inflammatory NLRs, such as NLRX1 and NLRP12, are the natural inhibitors of NF-κB that proficiently switch off the inflammatory cascade upstream of NF-κB signaling. Ligands for NLRX1 and NLRP12 have remained poorly described. However, a number of NLRX1 binding molecules and inhibitors were recently identified using a molecular docking approach to screen natural products and lipid databases (193). This study by Lu et al. revealed that punicic acid (PUA), eleostearic acid (ESA), and docosahexaenoic acid (DHA) can bind to the C-terminal fragment of the human NLRX1. Using $Nlrx1^{-/-}$ cells, the study showed that PUA and DHA suppressed the NF-κB activity in macrophages in a NLRX1-dependent mechanism *in vitro*. The NLRX1-dependent mechanism of PUA was further confirmed in the DSS model of colitis. In these studies, DSS-challenged mice were treated orally with either PUA (40 mg/kg body) or PBS. The WT mice treated with PUA showed significantly lower TNF-α and ameliorated mucosal inflammation compared to *Nlrx1*$^{-/-}$ counterparts (193). This study shows a great potential of NLRX1 in the treatment of inflammatory diseases.

Pidotimod (3-L-pyroglutamyl-L-thiazolidine-4-carboxylic acid) is a synthetic dipeptide immunomodulator that is largely used for treatment of respiratory tract infections, asthma, and chronic obstructive pulmonary disease (194). Previous studies show that pidotimod acts as an immunostimulant that induces DC maturation and T cell differentiation toward a T_H1 phenotype (195–197). A recent publication by Fogli et al. demonstrated the anti-inflammatory property of pidotimod in TLR-stimulated macrophages, which was associated with the increased expression of NLRP12 at both levels of mRNA and protein (198). Silencing NLRP12 expression recovered the proinflammatory response of pidotimod-treated cells, which suggests that the anti-inflammatory response of pidotimod was related to the levels of NLRP12 expression (198). These findings pave the way for the development of innovative treatments for inflammatory diseases through activating anti-inflammatory NLRs that naturally control the inflammatory pathways within cells.

PERSPECTIVES

Out of the 23 known NLRs in humans, only a handful of NLRs have been formally studied in MS to date. Given the prominent role of NLRs in host-pathogen interactions and inflammatory conditions, we anticipate that additional NLR signaling pathways will be found to impact neuroinflammatory diseases in the coming years. There are numerous NLRs that have been recently identified to affect inflammatory responses in other disease models [e.g., NLRP1, NLRP6, NLRC3, and NLRP4 (163, 199)] and we believe that it is only a matter of time until we come to fully appreciate the roles of these proteins in MS.

In recent years, there has been tremendous interest in the role that B cells play in MS due to the recent successes of anti-CD20-mediated B cell depletion in the treatment of both relapsing and primary progressive MS (200). B lymphocyte differentiation into plasma cells results in the secretion of immunoglobulins, which can bind and activate complement or induce antibody-dependent cytotoxicity (31). Surprisingly, little is currently known about NLR-dependent control of B cell responses in the context of demyelinating neuroinflammatory disease. Therefore, given the potent effect of NLRs on the homeostasis of the immune system, we expect that in the next few years MS research will focus on the role of NLRs in B cells.

Moreover, we may speculate that NLRs will emerge as attractive targets for therapeutic intervention in multiple neurological disorders, including MS, PD, AD, traumatic spinal cord, brain injury, and stroke.

AUTHOR CONTRIBUTIONS

MG and DG designed the format and figures and wrote the entire manuscript. TM contributed to the writing some sections of the manuscript including NLRP12 and innate immunity in the CNS. KG, AA, and JL contributed to the conceptual reading and critical editing of the manuscript. All authors read and approved the manuscript.

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Erratum: NLR-Dependent Regulation of Inflammation in Multiple Sclerosis

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Due to a typesetting error, the title was given incorrectly as "NLR-Dependent Regulation of Inflammation in Multiple Sclerosis- Dependent Regulation of Inflammation in Multiple Sclerosis." The correct title is "NLR-Dependent Regulation of Inflammation in Multiple Sclerosis."

The publisher apologizes for this error and the original article has been updated to reflect this. This error does not change the scientific conclusions of the article in any way.

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Microglia and CNS Interleukin-1: Beyond Immunological Concepts

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Activation of microglia and expression of the inflammatory cytokine interleukin-1 (IL-1) in the CNS have become almost synonymous with neuroinflammation. In numerous studies, increased CNS IL-1 expression and altered microglial morphology have been used as hallmarks of CNS inflammation. A central concept of how CNS IL-1 and microglia influence functions of the nervous system was derived from the notion initially generated in the peripheral immune system: IL-1 stimulates monocyte/macrophage (the peripheral counterparts of microglia) to amplify inflammation. It is increasingly clear, however, CNS IL-1 acts on other targets in the CNS and microglia participates in many neural functions that are not related to immunological activities. Further, CNS exhibits immunological privilege (although not as absolute as previously thought), rendering amplification of inflammation within CNS under stringent control. This review will analyze current literature to evaluate the contribution of immunological and non-immunological aspects of microglia/IL-1 interaction in the CNS to gain insights for how these aspects might affect health and disease in the nervous tissue.

Keywords: cytokine, neuroinflammation, brain, neuromodulation, IL-1R1

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INTRODUCTION

Changes in microglial morphology are one of the most common findings of neuropathology in almost all CNS diseases. Long regarded as the resident immune cells in the immunologically temperate environment of the CNS, the resting spider-shaped microglia become deramified and amoeboid in activated states (1). This shape shift has been observed in acute brain injury (2), various neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease (3), CNS autoimmune diseases such as multiple sclerosis (MS) (4, 5), convulsive disorders such as epilepsy (6), and even affective disorders including major depression (7, 8), anxiety disorders (9), and autism (10). These evidences led to the hypothesis that microglial activation is a significant common cause of neuropathology in these diseases (11), although microglial morphological changes alone may not always reflect the precise activation status (12, 13) among the variegated states that these cells can adapt.

Another salient-related observation in CNS diseases is the increased expression of the inflammatory cytokine interleukin-1 (IL-1). IL-1 is a master regulator of inflammatory reactions in the immune system, capable of activating innate immunity by inducing the expression of numerous inflammatory cytokines and chemokines, eliciting leukocyte infiltration into the inflammatory loci, increasing phagocytic and bactericidal activity of immune cells, enhancing activity of the complement system, and facilitating the activation of the adaptive immune responses (14). Correlations of plasma or CNS levels of IL-1 and disease severities in the abovementioned CNS diseases have been widely reported (15–20), although there are also many reports that fail to show correlation between plasma IL-1 level and the presence of disease symptoms in these diseases (21, 22).

Combining increased IL-1 expression and microglial activation as a composite indicator of pathogenesis in CNS diseases seems to be an attractive idea because it might overcome the shortcomings

of using each of them separately. In peripheral tissues, increased IL-1 expression is tightly linked with macrophage activation during inflammation (23); in the CNS, neuroinflammation may not display the entire panoply of peripheral inflammation, e.g., swelling may not occur during neuroinflammation, but increased expression of IL-1 by brain tissue together with morphological changes in microglia appear to be a frequently observed phenomenon in both human neuropathology and animal models of brain diseases (16, 24–28). Adding increased brain IL-1 expression to supplement microglial morphology changes would further specify that the changes in microglia are part of the inflammatory microgliosis.

The villainization of microglial activation and CNS IL-1 expression, however, has been countered by the teleological argument: is the CNS designed to have microglial activation and IL-1 expression just to cause pathology (29)? Such incredulity has been substantiated by the facts that blocking inflammatory microglial activation can lead to the exacerbation of some symptoms of certain CNS diseases (30–34) and clinical benefits of drug treatments for reducing microglial activation or IL-1 activity have been demonstrated recently in stroke patients (35), but the utility of this strategy for the treatment of the vast majority of the above-mentioned diseases remains to be firmly established after it has been advocated for at least 10 years.

Besides their pathogenic roles, functions of microglial activation and IL-1 expression in CNS development, repair, and physiological activity have been intensely studied recently. This endeavor has yielded tremendous advances, revealing many new areas of understanding on the non-immunological functions of IL-1 and microglia in the CNS (11, 36–39). These new findings in the realm of the positive contributions of microglia and IL-1 in the CNS educe the critical inquiry: how would the immunological and the non-immunological aspects of IL-1 and microglial functions coordinate or disrupt each other to affect health and disease?

THE PROMISES AND LIMITATIONS OF THE INFLAMMATORY PARADIGM

Although current literature is beginning to shed light on the multifaceted roles played by microglia and CNS IL-1, the simple inflammatory paradigm, *viz.*, increased CNS IL-1 expression together with microglial activation amplifies neuroinflammation and causes neuropathology, has accrued formidable experimental support. The following rationales have propelled the research in this area: (1) inflammatory process is designed to sequester and kill infectious pathogens and contain necrotic tissue damage; this entails the induction of proinflammatory cytokines and chemokines, recruitment of leukocytes, and the production of bactericidal reactive oxygen species (ROS), all potentially neurotoxic, (2) CNS is immunologically privileged site, bystander neuronal casualty from inflammation is likely to cause irreversible damage to this delicate tissue which lacks significant regenerative potential and expandable volume, and (3) neuroinflammation could lead to CNS autoimmunity resulting in attacks by immune cells to CNS antigens which are normally dormant.

The induction of CNS expression of proinflammatory cytokines including IL-1 has been shown in animal models of acute brain injury (40–43), Alzheimer's disease (44, 45), Parkinson's disease (25), CNS autoimmunity (46), anxiety disorder (47–50), major depression (51–53), and autism (54). *In vitro* studies were the first to show that inflammatory cytokines, especially IL-1 and TNF-α, can cause neuronal death by the direct effects of these cytokines on neurons or indirectly by glial production of neurotoxic substances (55–58). Similarly, a few chemokines have also been found to possess neurotoxic activity. CXCL4 (59), was the first to be identified in this regard; more recent studies also found CCL11 (60), CXCL2 (61) can exert neurotoxic effects on cultured neurons.

Neurotoxicity from infiltration of peripheral leukocytes has also been documented. Typically, in experimental conditions that resulted in leukocyte infiltration into the brain, the infiltrated peripheral myeloid cells show higher expression levels of proinflammatory cytokines than resident glial cells (62–64). Thus, entrance of peripheral leukocytes into the CNS may represent a more severe type of CNS inflammation. Reduction of leukocyte infiltration by blocking vascular adhesion molecules or chemokine activity has been shown to improve outcomes in acute brain injury (65, 66) and CNS autoimmune diseases (67, 68). Interestingly, although infiltration of peripheral leukocytes into the CNS is generally not a common observation in human affective disorders, this phenomenon occurs in several animal models of stress- or inflammation-induced depression and/or anxiety (69, 70). Preventing CNS infiltration of IL-1 expressing leukocytes protected animals from displaying depressive and/or anxiety-like behaviors in these models (64, 71).

Other studies demonstrated a pathogenic role of oxidative stress. Blocking inflammation-induced production of ROS or ROS activity alleviates neural damage in cerebral ischemia (72–74) and cerebral hemorrhage (75), reduces depressive and anxiety-like behaviors caused by peripheral inflammatory stimulation (76), lessens certain symptoms induced in an Alzheimer's mouse model (77). In addition, ROS production and antioxidant defense imbalance has been observed in acute brain injury (78, 79), inflammation-induced depression and anxiety, and neurodegenerative diseases (80, 81). These evidences support the hypothesis that oxidant/antioxidant imbalance downstream of IL-1-stimulated microglial activation is a common feature for both acute and chronic neuropathology and their attendant psychopathology (82, 83).

The possibility of bystander damage of CNS inflammation is best demonstrated in situations of CNS infection. Initially, post-infectious neurological dysfunction was thought as a consequence of permanent damage caused by the invading pathogens and the specific immune responses to the pathogen (84). However, patients who survived CNS infection sometimes show deficits implicating brain regions beyond the foci of the initial infection (85) and animal studies show chronic neuroinflammation may persist after the acute infectious pathogens have been eradicated (86). Thus, off-target inflammatory activity may contribute to post-infectious neuropathology.

Further bolstering the case for malignant inflammatory effects are the findings that endogenous CNS antigens that normally do

not induce autoimmune attacks can be turned susceptible when CNS inflammation is present. In experimental autoimmune encephalitis (EAE), the brain endothelial receptor for IL-1 (IL-1R1) and infiltration of myeloid cells expressing IL-1β was found to be required for the induction of illness (63). Because IL-1β-expressing myeloid cells are involved in inflammatory activity, not antigen specific immunity, these results point to the importance of inflammation in facilitating autoimmune activity of the CNS. Dysregulation of microglia may also contribute to the pathogenesis of PANDAS (Pediatric Autoimmune Neuropsychiatric Disorders Associated with Streptococcal Infections) which was thought to be caused by the induction of post-infectious cross-reactive autoantibodies against CNS tissue (87–89). Therefore, neuroinflammation might augment autoimmune activity-related neuropathology.

A major recent advance in the field of inflammation is the discovery of inflammasomes. Inflammasomes are protein complexes that act as intracellular sensors for the disruption of homeostasis (90). They include NOD like receptors and ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain). Inflammasomes regulate IL-1 and IL-18 activity by regulating caspase-1, which cleaves inactive pro-IL-1 and pro-IL-18 to derive the active IL-1 and IL-18. This intermediate step allows preformed pro-IL-1 and pro-IL-18 to be quickly activated, ensures inflammation occurs through the priming stage (the synthesis of pro-IL-1 and molecules of the Inflammasomes) and the activation stage (the generation of mature inflammatory cytokines), thus providing a mechanism that requires "two-hit" to induce inflammation, allowing finer control of the timing and the magnitude of inflammatory cascade. In addition, inflammasomes are sensitive to stimulations by internal disturbance, such as misfolded or aggregated proteins and aberrant products of energy metabolism, broadening the range of inflammation inducers beyond infectious stimuli (90). In ischemic or hemorrhagic stroke models, expression of the NLRP3, a known microglial inflammasome (91, 92) component, was increased, and specific blockade of NLRP3 reduced stroke induced neural damage and functional deficits (93, 94). Several NLRP3 component proteins were also induced in the pathological tissues in Alzheimer's disease (95). Aggregated or fibrillary α-synuclein, a known pathogenic factor for Parkinson's disease also stimulates the activation of NLRP3 (96). Activation of NLRP3 has been documented in depression and anxiety and both pharmacological blockade of NLRP3 or gene deletion of NLRP3 reduces depressive behavior and anxiety in animal models of these disorders (97, 98).

The inflammatory paradigm, increased brain IL-1 expression and microglial activation drives the progression of CNS diseases, has gained further momentum from studies that used drugs to inhibit IL-1 activity and/or microglial activation. A naturally occurring antagonist for IL-1 is the IL-1 receptor antagonist (IL-1ra). A recent meta-analysis shows treatment with IL-1ra reduces infarct volume by 36% in animal models of cerebral stroke with more reliable efficacy if the drug is delivered into the cerebral ventricle than into the blood (99). IL-1ra was also effective in blocking stress-induced depression and anxiety (51, 52, 100), and in improving clinical outcomes in experimental epilepsy (101, 102). Minocycline, a tetracycline derived antibiotics, has been found to inhibit inflammatory microglial activation (103). Specifically, activated microglia could differentiate into multiple activated states: the most inflammatory type is designated as M1 and the most anti-inflammatory type is designated as M2. Besides changes in morphology, M1 microglia express inflammatory cytokines including IL-1, TNF-α, and iNOS, whereas the M2 microglia express TGF-β, IL-4 or IL-10, and arginase 1. Treatment with minocycline selectively inhibits M1 microglial activation (104). Pretreatment with minocycline provides neuroprotection against excitotoxicity (105), oxidative stress (106), reduces symptoms in animal models of Parkinson's disease (107), cerebral stroke (108), epilepsy (109), and stress-induced depression (110). In EAE, a model of MS, minocycline treatment was found to be effective in reducing disease severity and histological outcomes when used in combination with other conventional treatments (111–116) or alone (117, 118). The promise of using drugs against IL-1 and microglial activation to treat CNS diseases is attested by the current clinical trials that use IL-1ra to treat cerebral stroke (119–121), fatigue in Sojegren's syndrome (122), and minocycline to treat cerebral stroke (123, 124), cerebral hemorrhage (125), Parkinson's disease (126, 127), epilepsy (128), bipolar and treatment resistant depression (129, 130), and schizophrenia (131). These trials have generated promising results, although large scale clinical tests are still needed. In human MS trials, minocycline treatment reduced MS lesion detected by MRI (132) and reduced the risk of conversion of patients with first demyelinating event from progressing to MS (133).

The notion that all CNS diseases can be effectively treated by inhibition of IL-1 driven microglial activation, of course, is overly simplistic. A dramatic cautionary tale is supplied by a study that investigated the role of IL-1β in Alzheimer's disease. Beta-amyloid aggregation in this disease causes the formation of senile plaques. Transgenic overexpression of IL-1β unexpectedly reduced plaque formation, despite inducing robust neuroinflammation (134). In another surprising study, chronic unpredictable stress induced depressive-like behavior; stimulating rather than inhibiting microglia provided anti-depressant effects (135). These results highlight the limitation of the inflammatory paradigm and suggest non-immunological functions of IL-1 and microglia should be examined.

IL-1 AND MICROGLIA AS NEUROMODULATORS

The neurophysiological functions of IL-1 were first investigated in temperature-sensitive neurons because IL-1 was identified as the endogenous pyrogen that mediates fever after bacterial infection. In the temperature control center of the brain, the preoptic area of the hypothalamus, IL-1 decreased the sensitivity of warm-sensitive neurons, but increased the sensitivity of cold-sensitive neurons, thereby modulating the thermoregulatory circuits in a manner consistent with its pyrogenic role (136, 137). This IL-1 activity is not related to neuroinflammation but could be an indirect effect because it can be blocked by inhibitors of cyclooxygenase, which catalyzes prostaglandin production downstream of IL-1 signaling (138). IL-1 may even mediate neurophysiological

effects under sterile condition. A good example here is its role in regulating normal sleep. IL-1 is expressed in the brain with a diurnal rhythm, and increased expression of IL-1 is associated with increased spontaneous sleep whereas inhibition of IL-1 activity reduces sleep (139). Interestingly, neuronal IL-1 expression and indirect activation of neurons by CNS IL-1 may underlie the sleep promoting effects of IL-1 as it can promote synchronization of sensory neurons (140). Other indirect electrophysiological effects of IL-1 have been reported in neurons of the supraoptic (138) and paraventricular nucleus of the hypothalamus (141). Direct effects of IL-1 on neuronal excitability have also been reported (138); but the mechanism for this function remains unclear. IL-1 was found to inhibit Ca+ channel currents (142), reduce GABA A receptor-mediated response (143), inhibit NMDA receptor-mediated synaptic transmission (144), activate non-selective cationic conductance (145), potentiate voltage-dependent sodium currents in nociceptive neurons (146, 147), and increase voltage-gated potassium currents (148), depending on the different types of neurons studied. In the dentate gyrus, IL-1 may facilitate or inhibit the generation of long-term potentiation (LTP) (149, 150), a critical neural mechanism for learning and memory. LTP occurs as persistent increases of synaptic strength after high-frequency synaptic stimulation, thus potentially coding for learning or memory processes. Interestingly, the learning process itself causes hippocampal expression of non-inflammatory levels of IL-1, which in term, helps maintain LTP (150, 151). These scattered reports of IL-1-mediated neurophysiological effect appear incongruent at first glance, but an emerging theme is IL-1 can modulate sensory system of the nervous system in order to modulate perception and learning. It should be noted that such modulation may have time-dependent and concentration-dependent variable effects. Acute IL-1 effects may heighten perception and learning whereas chronic IL-1 effects may reduce sensory function, retard learning, and cause fatigue (147, 148, 152–154). Similarly, low levels of IL-1 may facilitate memory whereas high levels of IL-1 or complete blockade of IL-1 signaling may impair memory (155). One difficulty in the past is to identify IL-1 receptor expressing neurons and the observed neuromodulatory effects of IL-1 may be attributed to the indirect action of IL-1 that might elicit neural active substances such as nitric oxide (156), ATP (157), or prostaglandins (145). Recently, we have developed a knockin mouse line that allowed the tracking of IL-1 receptor expression cells in a cell type specific manner. We now have unpublished results that show IL-1 type 1 receptor is preferentially expressed in numerous sensory brain regions.

Another neuromodulatory role of IL-1 is on neurogenesis. Reduced production of new neurons in adult hippocampus has been linked with the pathogenesis of depression (51). This role of IL-1 was initially observed in animal models of interferon-γ (IFN-γ) treatment. IFN-γ is used to treat hepatitis C but has the unfortunate side effect of causing depression. In a rat model of IFN-γ-induced depression, hippocampal IL-1β expression and reduced neurogenesis in dentate gyrus was induced and administration of IL-1ra blocked these effects together with the depressive behavior (158). This mechanism is also operative in chronic stress induced depression: chronic mild stress was found to induce IL-1β expression in the hippocampus, reduce neurogenesis, and cause depressive like behavior in wild-type mice. These changes were absent in IL-1 receptor knockout mice or transgenic mice that express IL-1ra in the brain (159). That IL-1 driven microglial activation may be involved in this phenomenon is further supported by the evidence that inhibition of NFκB activation blocked the antineurogenic and depressive effects of the stress (160). Brain IL-1 is known to induce microglial NFκB activation (161). It should be noted that chronic mild stress dose not induce leukocyte infiltration into the brain; thus this IL-1-mediated microglial activation may not represent an immunological neuroinflammation. In addition, the antineurogenic effect of IL-1 may also be concentration dependent as IL-1 can facilitate neuronal survival by promoting the expression of nerve growth factors (NGFs) (162).

Induction of neurotrophic factors is one of the early observations on IL-1-mediated non-immunological neural effects (163, 164). In traumatic brain injury, increased NGF expression follows the increased expression of IL-1 in the wounded tissue. Injection of IL-1ra blocked NGF and the associate neuroreparative responses (165). IL-1 has also been found to stimulate neurotrophin-3 and brain derived neurotrophic factor, supporting neuronal survival and neurite growth (166, 167). However, interaction between IL-1 and the neurotrophic factors can also be a double-edged sword. Systemic IL-1, not central IL-1, have been reported to reduce hippocampal BDNF expression (168); while acute intracerebral IL-1 caused the expression of neurotrophic factors and neuroprotection, subacute IL-1 (4 days of IL-1 injection) caused the opposite effects (169); IL-1 can also increase neuronal vulnerability by increasing the surface expression of the p75 neurotrophin receptor (170).

Physiological activities of IL-1 in the brain also include neuroendocrine functions. Psychological and metabolic stress induced ACTH and glucocorticoid responses were reduced in IL-1 receptor knockouts or transgenic mice overexpressing brain IL-1ra (171). Intracerebral administration of IL-1 is known to induce CRH release (172) and psychological stress has been shown to induce brain IL-1 expression (173). Therefore, brain IL-1 could mediate physiological response to stress by stimulating the production of the immunosuppressive hormone glucocorticoid. In addition, IL-1 acting in the brain can stimulate brain metabolism despite hypoglycemia. Neuronal IL-1 synthesis was found to be induced by stimulation of AMPA receptors on neurons and the resulting release of IL-1 can stimulate glucose uptake by neurons in an autocrine or paracrine fashion (174). It is interesting to speculate that these physiological activities of IL-1 might coordinate with the immune activities of IL-1 such that hyper-inflammation may be prevented and brain energy usage may be spared even when immune activity might be energetically costly. It is interesting to note that the neuroendocrine function of IL-1 may be evolutionary conserved from invertebrates. In molluscs, CRF causes the production of biogenic amines as a stress response. This response is significantly reduced by IL-1 (175). Thus, this non-immunological IL-1 activity may have an ancient origin.

The non-immunological activities of microglia have been reviewed extensively. The readers are referred to these excellent reviews (11, 36, 176–178). Briefly, emerging evidences show microglia perform surveillance function during "resting state," prune excessive synapse during development, contribute to adult

neurogenesis, support neuronal survival, and modulate neurotransmission. Current research using advanced techniques in molecular biology, imaging and immunology has also identified significant heterogeneity in brain microglia in terms of morphology, gene expression profile, and cellular origin and fate (179). Some characteristics of subsets of microglia appear to be tightly linked with the potential neural function of these cells. For example, microglia from neurogenic regions are capable of substantial proliferation whereas microglia from non-neurogenic regions are not (180). Analysis of microglial expression patterns suggests that microglia from cerebellum and hippocampus appear immunologically more vigilant than microglia from other brain regions. Within Basal ganglia, microglia were found to show regional specific morphology, cell number, expression profile and activity relevant to motor activity and motion control, shaped by local cues (181). These findings demonstrate non-immunological functions of the microglia could be influenced by the specific neural circuitry they modulate. From this perspective, it is interesting to note that chronic unpredictable stress causes depression in association with a reduction of microglia numbers in hippocampus and stimulation of microglial activation by LPS or M-CSF restored microglia numbers and ameliorated stress-induced depression. In another study, chronic unpredictable stress was found to activate microglial cells in association with elevated CSF-1 expression in the prefrontal cortex (PFC), increase microglial phagocytosis of neuronal elements, and reduce dendritic spine density. Viral vector mediated knockdown of CSF-1 in the PFC blocked these effects and stress-induced anxiety- and depressive-like behavior (182). In neurodegenerative disease models, microglial production of proinflammatory cytokines and growth factors has been found to mediate neuroprotection against excitotoxicity (183, 184). In addition, microglia-mediated synaptic stripping was found to be neuroprotective following acute neural injury (185, 186). On the other hand, abnormal synaptic pruning have been observed in obsessive-compulsive disorder (OCD), indicating this mechanism might be pathogenic in OCD (187). These findings show non-immunological activities of microglia can be either neuroprotective or pathogenic depending on the specific circumstances.

THE BIG PICTURE

The dizzying progress made in the field of CNS IL-1 and microglia has produced great excitement and confusion. It is clear CNS IL-1 and microglia have both immunological and non-immunological functions. These two types of functions may be separated not only by the physical barrier, such as the blood brain barrier, but also by an invisible barrier: the activation threshold of inflammatory cytokines. For example, IL-1 is able to activate neurons at 1,000-fold lower concentration than that is required for the activation of non-neuronal cells (188). It is possible that low levels of IL-1 acts in the CNS to perform non-immunological functions including non-immunological activation of microglia, which are involved in the remodeling of the CNS tissue. Higher concentration of IL-1 could engage non-neuronal cells of the CNS to produce neuroinflammation. Interestingly, although microglia is the main source of IL-1 production in the brain without infiltrated leukocytes, IL-1 does not directly stimulate microglial cell to produce IL-1 (189). Our unpublished results show IL-1 receptor is not expressed on resting microglia and CNS IL-1 induce microglia to produce IL-1 indirectly *via* cells of the blood-brain barrier and cells of CSF-brain barrier. The separation of the immunological and non-immunological functions of CNS IL-1 and microglia may be compromised during neural injury or aberrant neural activity. Thus the integrated perspective suggests that the disruption of the proper separation and coordination of the immunological and the non-immunological functions of CNS IL-1 and microglia might be a new way to think about the pathogenic potential of these two critical factors in CNS diseases.

Another important insight is that the detrimental effects of IL-1 and microglial activation does not always stem from immunological functions of these factors. A series studies from Centonze's group showed that IL-1 and TNFα can cause hyperexcitation in neurons, causing excitotoxicity in MS (190). In addition, they found IL-1 could cause anxiety by blocking neuronal cannabinoid receptor 1-mediated control of GABAergic synapses (49, 100, 191, 192). Thus, aberrant non-immunological function of IL-1 can also contribute to disease progression.

The complex contribution of CNS IL-1 and microglia argues against a one size-fits-all approach to target these factors in treatment without careful considerations for the different phases of pathological processes. For acute brain tissue injury, blocking IL-1 activity and microglial activation at the early phase of the disease could be beneficial as this might dampen excessive neuroinflammation (15, 193); however, blocking later expression of low levels of IL-1 related to its promotion of clearing of debris and wound healing (194) may not be advisable. In chronic degenerative diseases, blockade of CNS IL-1 activity and microglial activation may also need to be titrated, such that the excessive activation of these factors may be attenuated, but

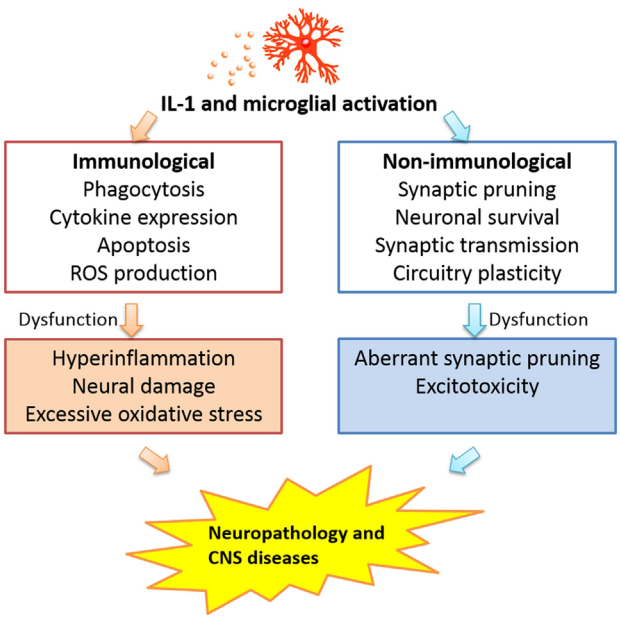

FIGURE 1 | Interleukin-1 activity and microglial activation may influence CNS pathogenesis *via* dysregulation of either the immunological or the non-immunological functions of these factors.

the physiological neuroregulatory functions can be preserved. In an extreme case of an animal model of major depression, loss of microglia in hippocampus has been found to be a cause and stimulation of microglia proliferation can effectively alleviate behavioral symptoms of depression (195). Thus, one cannot assume that microglial activation, not deficiency, is always the cause of CNS diseases. The integrated perspective of microglial activation and IL-1 activity in the brain in regards to the pathogenesis of CNS diseases is presented in **Figure 1** in which the immunological and non-immunological functions of IL-1 and microglial activation are seen as an integrated whole and dysregulation of either types of functions alone or in combination may contribute to disease progression.

AUTHOR CONTRIBUTIONS

NQ and XL cowrote this review.

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Significance and *In Vivo* Detection of Iron-Laden Microglia in White Matter Multiple Sclerosis Lesions

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Microglia are resident immune cells that fulfill protective and homeostatic functions in the central nervous system (CNS) but may also promote neurotoxicity in the aged brain and in chronic disease. In multiple sclerosis (MS), an autoimmune demyelinating disease of the CNS, microglia and macrophages contribute to the development of white matter lesions through myelin phagocytosis, and possibly to disease progression through diffuse activation throughout myelinated white matter. In this review, we discuss an additional compartment of myeloid cell activation in MS, i.e., the rim and normal adjacent white matter of chronic active lesions. In chronic active lesions, microglia and macrophages may contain high amounts of iron, express markers of proinflammatory polarization, are activated for an extended period of time (years), and drive chronic tissue damage. Iron-positive myeloid cells can be visualized and quantified with quantitative susceptibility mapping (QSM), a magnetic resonance imaging technique. Thus, QSM has potential as an *in vivo* biomarker for chronic inflammatory activity in established white matter MS lesions. Reducing chronic inflammation associated with iron accumulation using existing or novel MS therapies may impact disease severity and progression.

Keywords: multiple sclerosis, microglia, magnetic resonance imaging, quantitative susceptibility mapping, myelin, iron

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INTRODUCTION TO MICROGLIA

Microglia are resident immune cells of the central nervous system (CNS) responsible for homeostatic functions, including neurogenesis and clearance of cellular debris, and for responding to injury and infection (1–3). In a resting state, microglia have a ramified appearance with thin processes that survey the surrounding microenvironment (4–6). Following activation, microglia and macrophages can adopt a spectrum of phenotypes composed of pro-inflammatory (M1) and anti-inflammatory (M2) functions (7–11). The classically activated M1 phenotype is characterized by expression of pro-inflammatory cytokines (e.g., IL-1β, TNF-α), and induction of nitric-oxide synthase (6, 12, 13), while the M2 phenotype is characterized by secretion of anti-inflammatory cytokines (e.g., IL-10), and neurotrophic and angiogenic factors (6, 12, 13). Changes in activation status and cell signaling induce morphological changes, motility, and phagocytosis (14). Even though microglia and macrophages express similar cell surface markers and can be morphologically indistinguishable (13, 14), they originate from distinct progenitors: macrophages are monocyte-derived, while microglia arise from differentiated yolk sac erythromyeloid precursors (15–17). Macrophages have been widely studied *in vivo* and *in vitro*; however, the functions of microglia are still not well defined, including their roles in inflammatory diseases such as multiple sclerosis (MS).

MS LESION PATHOLOGY

Multiple sclerosis is a chronic inflammatory disease of the CNS, characterized by focal demyelination, that is caused by an autoimmune response to self-antigens (18). MS is the most common cause of non-traumatic neurological disability in young adults, affecting more than 2.3 million people worldwide. The disease usually starts with episodes of neurological dysfunction that remit spontaneously, a course that is termed relapsing remitting MS (RRMS). One to two decades into RRMS, most MS patients enter a secondary progressive phase, where relapses are replaced by slow, irreversible progression of neurological disability (19). Significant strides have been made in understanding the pathophysiology of relapses; however, progression remains largely unexplained. Multiple lines of evidence suggest that progressive MS is associated with chronic activation of the CNS innate immune system (20–22).

Inflammatory demyelinating lesions are a pathological hallmark of RRMS. Acutely demyelinating lesions are characterized by a breach of the blood–brain barrier (BBB), infiltration with leukocytes, and breakdown and phagocytosis of myelin (23). Acute lesions evolve into chronic active lesions, which contain a demyelinated, gliotic lesion center, and activated microglia and macrophages at the lesion edge. Depending on the activation status and phagocytotic activity of myeloid cells at the lesion rim, chronic active lesions may stay dormant or continue to slowly expand (**Figure 1**) (24). Eventually, chronic active lesions become chronic silent, i.e., they no longer contain inflammatory cells (25). Myelin-laden, foamy macrophages in the center and inner rim of acute lesions express anti-inflammatory cytokines (26), suggesting that myelin phagocytosis induces an anti-inflammatory phenotype, which may contribute to the eventual resolution of inflammation. The M2-inducing properties of myelin uptake have been confirmed in cultured monocyte-derived macrophages and in mouse models of spinal-cord injury (26–29).

A more recent study posits that myelin-containing macrophages in actively demyelinating areas exhibit a mixed phenotype expressing both M1 and M2 markers, including CD40, CD86, CD64, and CD32 (M1), as well as mannose receptor and CD163 (M2) (30). Moreover, at the rim of chronic active lesions, microglia lack expression of M2 markers, suggesting that the M2 component becomes extinguished once the lesion progresses from acutely demyelinating to chronic active (30). M2 markers are also expressed by microglia and macrophages during remyelination (31).

THE ROLE OF IRON IN THE CNS

A striking feature of chronic active MS lesions is that iron is highly enriched in activated microglia and macrophages at the lesion edge (27), which has implications for their function and *in vivo* detection in MS patients, as discussed below. Iron acts as a cofactor for various enzymatic reactions, and is essential for normal brain function, specifically the synthesis and maintenance of myelin (32, 33). Accordingly, in the CNS, iron is present primarily in oligodendrocytes and myelin (34), where it is stored predominantly in the redox-inactive ferric (Fe^{3+}) form within ferritin. Unbound ferrous iron (Fe^{2+}) can catalyze production of reactive oxygen species (ROS) through the Fenton reaction (35, 36). This cytotoxic process is minimized through a highly coordinated process that involves specific iron transport, uptake, and storage proteins, including transferrin, transferrin receptor, hepcidin, divalent metal transporter 1, ferroportin, and ferritin (37, 38).

In the normal aging brain, iron levels increase in the cortex, cerebellum, and deep gray matter (39, 40). Accelerated accumulation of iron in the basal ganglia and motor cortex have been demonstrated in several CNS disorders such as Parkinson's disease (41), Alzheimer's disease (42), Huntington's disease (43, 44), amyotrophic lateral sclerosis (45), and MS (46–52). While iron accumulation correlates with disease progression, the pathological processes have not been well delineated. Iron accumulation may be associated with excess ferrous iron and ROS production (53), but it is unknown whether iron accumulation is the cause

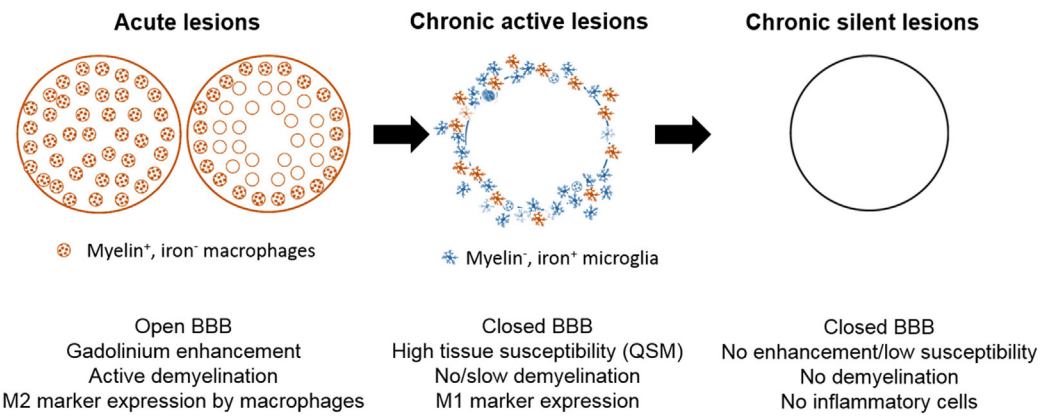

FIGURE 1 | Schematic of white-matter lesion development and its representation with quantitative susceptibility mapping (QSM). Acutely demyelinating lesions are Gd enhancing on T1w imaging and contain M2-activated macrophages. Acute lesions eventually progress to chronic active lesions that may contain iron+ microglia/macrophages at the lesion rim and express M1 activation markers. These lesions are typically non-enhancing and appear hyperintense with respect to normal appearing white matter (NAWM) on susceptibility weighted imaging. Chronic silent lesions lack inflammatory cells and their susceptibility is similar to that of nearby NAWM.

of tissue damage or occurs secondary to neurodegeneration. Oligodendrocytes, oligodendrocyte progenitors, and neurons are particularly sensitive to ROS, as they are unable, unlike astrocytes, to produce high levels of the free-radical scavenger glutathione (54–56). Glutathione also inhibits an iron-dependent form of programmed cell death, ferroptosis, triggered by iron overload (57–60). Furthermore, high iron induces glutamate release by neurons (61, 62), which potentially leads to excitotixicity in neurons and oligodendrocytes.

In MS, increased iron in deep gray matter has been inferred from T2 hypointensities on magnetic resonance imaging (MRI), although changes in T2 signal can be caused by multiple factors, including inflammation and edema. Clinically, deep gray matter T2 hypointensities correlated with brain atrophy, disability progression, and cognitive impairment (47–50, 63). In a study that used quantitative susceptibility mapping (QSM) rather than T2 signal to map iron content, magnetic susceptibility in basal ganglia of MS patients correlated with decreased performance on basal ganglia–reliant neuropsychological tasks (64). Histologically, iron was present in deep gray matter primarily in oligodendrocytes and myelin fibers, and to a lesser extent, in microglia and astrocytes; In contrast to imaging studies, a statistically significant iron increase in deep gray matter of MS patients compared with controls could not be demonstrated (65).

IRON IS A MARKER OF CHRONIC INFLAMMATORY MS LESIONS

A second site of iron accumulation in MS is in activated microglia/macrophages at the rim of chronic active lesions (27, 66, 67). Myeloid cells play important roles in iron homeostasis, including iron recycling through erythrophagocytosis (68) and induction of inflammatory hypoferremia (69), which bolsters resistance to infectious diseases. Since microbes depend on iron for growth and survival, its sequestration by macrophages is an important inflammatory response (70, 71). In activated macrophages, accumulation of iron is promoted by IL-6 and IL-1β, which induce the iron regulatory hormone hepcidin (69, 72, 73). Thus, iron accumulation is partially regulated by pro-inflammatory cytokines, consistent with the observation that iron uptake correlates with functional polarization of macrophages/microglia. Classically activated (M1) macrophages *in vitro* take up more iron than M2 or M0 macrophages (27, 74, 75), in keeping with the low iron levels in myelin-laden, M2-polarized macrophages *in vitro* and in acutely demyelinating lesions (26, 27). We have recently confirmed that iron uptake is enhanced in human-induced pluripotent stem cell-derived microglia following M1 polarization (unpublished data). Moreover, iron induces a persistent pro-inflammatory state in macrophages in chronic venous ulcers and spinal-cord injury, thus preventing the physiologic switch from M1 to M2 activation associated with wound healing (74, 75). While the direct effects of iron accumulation on macrophage activation are not completely understood, one proposed mechanism is that high intracellular iron activates nuclear factor-kappa B (NF-κB), leading to expression of NF-κB target genes including pro-inflammatory cytokines (76). In additional preliminary data, we found that iron-positive, chronic active lesions contained substantially more activated microglia/macrophages that expressed iNOS, ferritin, and the phagocytosis marker, MerTK, compared with iron-negative, chronic active lesions.

The source of iron in MS lesions is unknown, but it is tempting to speculate that the destruction of iron-rich myelin and oligodendrocytes during lesion formation leads to iron release into the extracellular space and eventual uptake by myeloid cells. Hametner and colleagues have shown that iron is decreased in oligodendrocytes within NAWM in patients with longstanding disease (67), suggesting a shift of iron from oligodendrocytes to microglia, which may impair the ability of oligodendrocytes to maintain myelin or to remyelinate.

DETECTING CHRONIC INFLAMMATION IN MS PATIENTS

Magnetic resonance imaging is a valuable tool for diagnosing MS and monitoring inflammatory activity in MS patients. Acutely demyelinating lesions can be visualized through gadolinium that accumulates within lesions with temporary breakdown of the BBB (77–79). However, gadolinium enhancement offers only a small window into early inflammatory activity, as the BBB closes within weeks of lesion formation (**Figure 1**). Gadolinium enhancement in MS lesions is preceded and outlasted by infiltration with immune cells. This has been demonstrated in MS patients with positron emission tomography (PET) imaging studies using radioactive ligands for the 18-kDa translocator protein (TSPO) (80), and with MRI of ultra-small iron-oxide particles that were injected peripherally and detected in activated monocytes/macrophages infiltrating the lesions (81). These imaging results are consistent with histological studies indicating that significant inflammatory activity occurs behind a closed BBB (82).

The therapeutic goal of managing MS patients is to completely suppress CNS inflammation. Thus the inability to detect chronic inflammation in MS with conventional MRI techniques is a significant, unmet need in clinical practice. While TSPO-PET imaging allows for assessment of glial cell activation, PET imaging requires significant infrastructure, is costly, and involves patient exposure to radioactivity, all of which make this method unsuitable for broad use in clinical practice. A solution to the problem of visualizing activated microglia/macrophages in lesions is to exploit their high iron content using novel MRI techniques.

QSM IN MS

Tissue can become magnetized in response to a magnetic field, and the extent of magnetization is known as susceptibility, which arises from unpaired electrons in iron or external sources such as contrast agents. MRI permits visualization of tissue susceptibility through gradient echo (GRE) and phase imaging. These techniques have been used to monitor MS lesions (27, 66, 83, 84), but they cannot quantify or localize iron (85). QSM permits visualization of the sizes and shapes of iron sources, delivers precise estimates of iron concentrations, and distinguishes between susceptibility sources such as iron and calcification (85). QSM

maps both ferrous (Fe^{2+}) and the substantially more common ferric (Fe^{3+}) iron, but cannot distinguish between the two sources. In addition, the presence of lipid macromolecules such as myelin reduces tissue susceptibility, resulting in increased susceptibility in demyelinated lesions. QSM is now widely used by the imaging research community in applications to detect iron, map bone mineralization and monitor drug bio-distribution delivered by magnetic-core nanocarriers (38, 44, 86–103).

Several studies, including our own, combined QSM or phase imaging of MS autopsy tissue with histological analysis, and confirmed that high tissue susceptibility at the rims of MS lesions correlated approximately with the distribution of iron and $CD68^+$ microglia/macrophages (**Figure 2**) (24, 27, 66, 83, 104, 105), which contain predominantly ferric iron. In addition, elemental tissue analysis with laser ablation mass spectrometry combined with QSM and IHC of autopsied lesions has established that positive susceptibility values were associated with iron deposition in activated microglia/macrophages (104). In a separate study, X-ray fluorescence imaging and histochemical techniques on autopsied MS brains demonstrated that iron accumulated in microglia/macrophages in chronic lesions (106). These results demonstrate that white matter lesions with high tissue susceptibility at the lesion rim are indicative of iron-positive microglia/macrophages.

An unresolved challenge regarding QSM is the inability to distinguish between the contributions of iron accumulation and myelin loss to lesion susceptibility (107, 108). Both can cause an increase in susceptibility, which generates the need to develop a method to separate the two sources. Birkl et al. addressed the confounding effect of myelin on iron quantification in MS tissue by exploiting the temperature dependency of the susceptibility of paramagnetic iron, which decreases with temperature, while the susceptibility of the diamagnetic myelin remains constant (109). While this technique is well suited for *ex vivo* research, it cannot be applied to patients. In addition, a study of lesions in MS tissue by Wiggermann et al. (108) that determined the sources of lesion contrast on QSM, found a poor correlation between lesional iron content and QSM. While these findings may be explained in part by the low iron content in the examined lesions, their data suggest that the QSM contrast between lesions and the surrounding NAWM may be driven by pathological changes known to be present in NAWM. Therefore, using NAWM as susceptibility reference, as is common in current practice, can lead to an incorrect interpretation of QSM change. A more reliable reference is cerebrospinal fluid, which consists essentially of water and can provide a uniform zero-reference (110).

In the first study that applied QSM to MS, Langkammer et al. demonstrated in patients with established MS or with clinically isolated syndrome, an isolated MS-like neurological episode, that QSM is more sensitive than R2* in the detection of tissue changes in the basal ganglia (107). The authors interpreted the increase in susceptibility as a consequence of increased iron content, but noted that demyelination may play an additive role. In a small clinical imaging study, we demonstrated that patients with active RRMS contained significantly more lesions with high susceptibility on phase imaging than patients with chronic, stable disease (27). Furthermore, we found in a retrospective study, where susceptibility was quantified in white matter lesions of different ages, tissue susceptibility was isointense in Gd-enhancing lesions, and increased rapidly after enhancement subsided, suggesting that lesions acquired iron as they transitioned from an acute to a chronic active state. The elevated susceptibility was stable for approximately 4 years and then decayed to levels similar to that of NAWM (**Figure 1**) (111). This time course of tissue susceptibility was recently confirmed in a separate longitudinal study with MS patients (112). On a cellular level, the isointense susceptibility in enhancing lesions may be

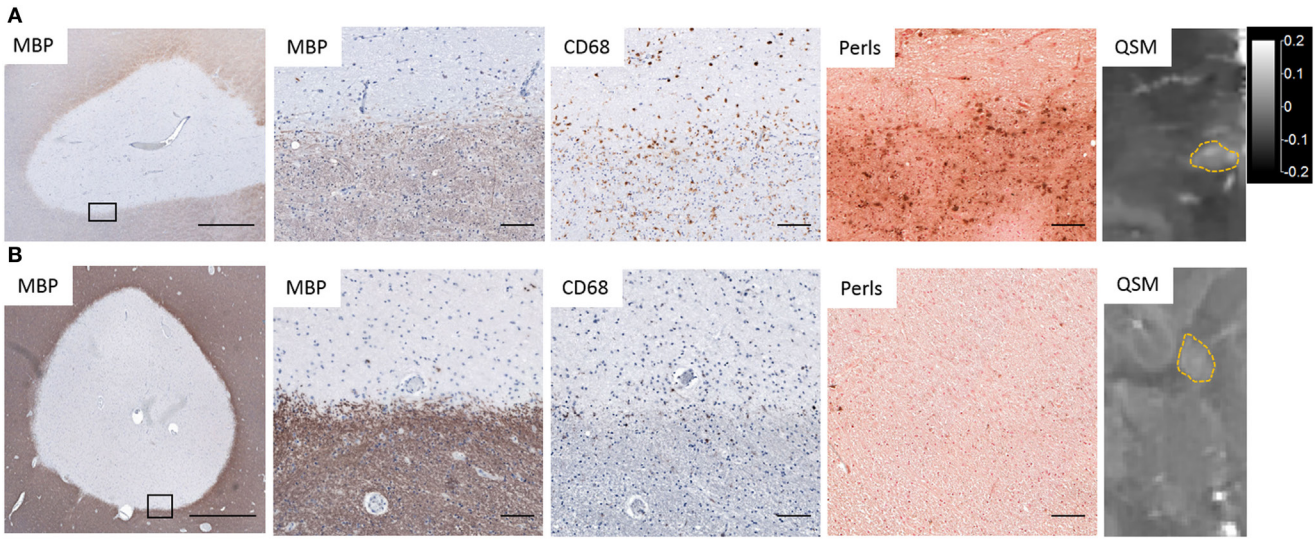

FIGURE 2 | Iron deposition in chronic active lesions corresponding to regions of hyperintensity on quantitative susceptibility mapping (QSM). **(A)** Iron-positive lesions contain $CD68^+$ $Perls^+$ microglia and macrophages at the lesion rim whose distribution corresponds to hyperintensities on QSM. **(B)** Iron-negative lesions contain few $Perls^+$ microglia and have low-tissue susceptibility on QSM. Black rectangles in low magnification images identify the location of higher magnification images. Scale bar in low magnification images = 1,000 μm. Scale bar in high magnification images = 100 μm. QSM scale bar is the same for both images and is in ppm (parts per million).

explained by the reduced capacity of myelin-phagocytosing macrophages to take up iron (27), consistent with the M2-like phenotype of myelin-laden macrophages (26). As the lesion evolves, myelin-laden macrophages continue to break down ingested myelin and eventually exit the lesion center. Activated non- or slowly phagocytosing myeloid cells at the lesion rim accumulate iron and adopt a chronic inflammatory state (75) that may persist for several years (111, 112).

In a recent prospective imaging study using phase imaging, persistence of phase rims in white matter lesions was associated with increased lesion T1 hypointensities, a marker for tissue damage (113). In addition, Dal-Bianco et al. reported that white matter lesions with phase-positive rims slowly expanded over time, supporting the idea that iron-positive microglia/macrophages are associated with chronic, slow inflammatory demyelination (24). It is tempting to speculate that high prevalence of lesions with hyperintense rims is associated with a more severe disease course and/or disease progression; however, these data are not yet available. Prospective studies examining these correlations are currently ongoing at our centers.

The prevalence of white matter lesions with hyperintense rims on QSM and phase imaging varies widely, ranging from 0 (113) to 32% (114). This variability is unsurprising given the different imaging techniques, resolutions, and patient cohorts used in these studies. An imaging study on MS patients from our group revealed that 21% of lesions visible on QSM had a hyperintense rim, and 79% displayed homogenous or heterogenous distribution patterns (115). Our preliminary data from a combined imaging and histology study of MS brain tissue suggest that heterogenous QSM patterns were typically associated with the presence of heme within enlarged blood vessels in MS lesions. We have currently no data to explain homogenously increased susceptibility throughout lesions, but hypothesize that absence of myelin drives the susceptibility increase in these lesions.

In summary, although susceptibility weighted imaging cannot distinguish between iron accumulation and myelin loss, increased susceptibility at the lesion rim likely represents chronically activated, iron-positive microglia and macrophages. Moreover, longitudinal imaging studies of MS patients using QSM suggest that iron-positive lesions persist for many years and are associated with increased tissue loss and slow expansion (24, 113).

CLINICAL IMPLICATIONS

Based on the above studies, high tissue susceptibility in white-matter lesions may be useful as a biomarker for chronic active lesions. Although the detrimental effect of smoldering, low-grade inflammation on the surrounding parenchyma has been demonstrated (24, 113), it is unknown if the presence of hyperintense susceptibility rim lesions predict a more severe clinical course; studies are ongoing that examine this association. Moreover, we are testing the ability of current MS treatments to remove iron from existing white matter lesions in MS patients. Of particular interest are MS medications that penetrate the BBB and act directly on microglia, such as dimethyl fumarate (Tecfidera™), fingolimod (Gilenya™), and Laquinimod (116–118).

Since QSM can be rapidly and reliably acquired with standard field strength (3T) MRI scanners, it can easily be implemented in clinical settings and broadly used for MS patient care. Thus, iron-sensitive imaging may become an important imaging modality to detect chronic inflammation in MS patients that appear stable on conventional MRI but have a high burden of lesional microglial activation.

SUMMARY AND OUTLOOK

We reviewed iron metabolism in macrophages/microglia, iron accumulation in MS lesions, and iron-sensitive imaging studies in MS tissue and patients. Iron is taken up by M1-polarized macrophages/microglia, which may further increase their pro-inflammatory properties. Iron can be visualized with MR sequences sensitive to tissue susceptibility. In MS patients, high susceptibility in white matter lesions can persist for several years after lesion formation, suggesting that iron-positive myeloid cells are present in MS lesions for prolonged periods of time. In addition, high susceptibility is associated with increase tissue loss and lesion expansion.

Therefore, the emerging picture suggests that iron-positive microglia and macrophages in chronic active MS lesions constitute a distinct, previously unappreciated inflammatory compartment that may be a significant contributor to tissue damage, disease severity, and/or progression. Reducing chronic inflammation associated with iron deposition in MS lesions with existing or novel MS therapies may be of high benefit to patients.

AUTHOR CONTRIBUTIONS

KG and DP wrote the manuscript. MM, KG, and TDN conducted experiments. All authors contributed to analysis and interpretation.

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REVIEW
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The Role of Astrocytes in Multiple Sclerosis

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The role traditionally assigned to astrocytes in the pathogenesis of multiple sclerosis (MS) lesions has been the formation of the glial scar once inflammation has subsided. Astrocytes are now recognized to be early and highly active players during lesion formation and key for providing peripheral immune cells access to the central nervous system. Here, we review the role of astrocytes in the formation and evolution of MS lesions, including the recently described functional polarization of astrocytes, discuss prototypical pathways for astrocyte activation, and summarize mechanisms by which MS treatments affect astrocyte function.

Keywords: astrocytes, multiple sclerosis, neuroinflammation, NF-κB, risk variant, leukocyte recruitment

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INTRODUCTION

Multiple sclerosis (MS) is an autoimmune disease that targets the central nervous system (CNS) (1). It is the most common, non-traumatic neurological disorder in young patients and affects nearly 1 million people in the US alone (2). In the majority of MS patients, the disease manifests itself as episodes of neurological dysfunction that remit spontaneously [relapsing–remitting MS (RRMS)] (1). Pathologically, relapses are associated with focal, inflammatory demyelination in white and gray matter, characterized by infiltration with macrophages and T and B lymphocytes (3). Over two-thirds of patients eventually develop secondary progressive MS (SPMS), a disease stage that is believed to be driven by neurodegeneration, where patients experience slow and irreversible accumulation of disability, predominantly affecting ambulation and cognition (1, 4). In a small percentage of MS patients, progression sets in at disease onset, a disease course that is termed primary progressive MS (1). The pathophysiology of primary and secondary progression remains largely unexplained; however, multiple lines of evidence suggest that progressive MS is associated with chronic activation of the CNS innate immune system (5–7). The poor understanding of the pathomechanisms underlying progression is reflected in the current treatment options for MS, with 13 FDA-approved medications being available for RRMS, one moderately effective medication for primary progressive MS, and none for secondary progression (8).

Multiple sclerosis is the result of an interplay between environmental and genetic factors. Genome-wide association studies (GWASs) have identified over 230 variants associated with susceptibility for MS that all confer small increases in disease risk (9–11). Environmental factors associated with MS risk include smoking, childhood obesity, low vitamin D levels, infection with the Epstein–Barr virus, and possibly a high salt diet (12–14). The pathological hallmark of MS is the presence of focal inflammatory lesions characterized by primary demyelination and relative preservation of axons (15). Acute demyelinating lesions are populated by abundant foamy, myelin-laden macrophages and by lymphocytes that are located in the perivascular space and diffusely throughout the lesion area, albeit at much lower numbers than myeloid cells (15). Acutely demyelinating lesions eventually evolve into chronic active lesions, which are characterized by completed demyelination and astroglial scarring in the lesion center, and inflammatory cells at

the lesion rim, possibly associated with low-grade demyelination (15). Finally, chronic silent lesions consist of astroglial scars with few or no inflammatory cells (15). Astrocytes have traditionally been assigned a bystander role, reacting only once demyelination is completed by forming a glial scar (16). However, recent literature suggests that astrocytes are early and highly active participants in MS lesion development (17–19). Astrocytes play multiple roles in the evolution of MS lesions, not only by recruiting lymphocytes (19, 20) and contributing to tissue damage (21–24) but also by confining inflammation and promoting lesion repair (18). In addition, astrocytes themselves sustain significant damage during the inflammatory process (16). This review focuses on the contributions of astrocytes to MS lesion formation. We discuss astrocytic phenotypes, prototypical pathways for astrocyte activation, including the impact of genetic risk variants for MS susceptibility on astrocyte responses, and mechanisms by which MS treatments affect astrocyte function.

THE PHYSIOLOGICAL ROLE OF ASTROCYTES AND ASTROCYTE RESPONSES

Astrocytes make up approximately 30% of glial cells in the CNS, where each astrocyte occupies a unique territory demarcated by non-overlapping, star-shaped processes that extend from the cell soma (25, 26). The distal end feet of these processes form the glia limitans when they envelop the parenchymal basal lamina associated with blood vessels or meninges (18, 25). The glia limitans contributes to the maintenance of blood–brain barrier (BBB) integrity and forms a secondary barrier that further restricts entry of peripheral immune cells into the CNS (16, 27). Astrocytes are paramount for normal CNS functions, including maintenance of glutamate, extracellular potassium, and water homeostasis (20, 25). Astrocytes are functionally connected to adjacent astrocytes and to oligodendrocytes by gap junctions, thereby forming large syncytium-like glial networks that are composed of hundreds of cells (28). Together with neuronal synapses, astrocyte processes form so-called tripartite synapses, where one single astrocyte connects with tens of thousands of neuronal synapses (29) to regulate neuronal synaptic transmission, e.g., by releasing glutamate, D-serine, and ATP (30, 31). Astrocytes also prune synapses through phagocytosis (32) and modify gene expression, e.g., associated with neural plasticity, in surrounding neurons by secreting miRNA-containing exosomes (33). In addition, astrocytes secrete neurotrophic factors (34) and are metabolically coupled to neurons, releasing lactate for neuronal uptake and providing antioxidants such as glutathione and thioredoxin (35, 36). Astrocytes also participate in the production of neurosteroids, such as allopregnanolone, estrogen, and dehydroepiandrosterone (DHEA), that are synthesized in the nervous system, where they modulate neuronal excitability, promote myelination, and dampen pro-inflammatory responses in astrocytes (37–41). Moreover, in the healthy CNS, astrocytes contribute to an anti-inflammatory environment through constitutive low-level secretion of the anti-inflammatory cytokines TGF-β (42) and IL-10 (43), expression of Fas ligand (44, 45), and induction of upregulation of the co-inhibitory cell surface receptor CTLA-4 on helper T cells (46).

Astrocyte reactivity in adaptive and innate immune responses can be triggered through oxidative or chemical stress, pro-inflammatory cytokines, damage-associated molecular patterns (DAMPs), released in the context of CNS tissue damage, and pathogen-associated molecular patterns (PAMPs), such as double-stranded RNA and bacterial membranous endotoxins, released from pathogens (19, 47, 48). Stimulation of astrocytes induces or upregulates astrocytic secretion of cytokines, such as TNF-α, IL-1β, and IL-6; neurotrophic factors including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), vascular endothelial growth factor (VEGF), and leukemia inhibitory factor (LIF) (19, 48–50); chemokines including CCL2, CCL20, and CXCL10; and β-defensins, antimicrobial peptides that can directly diminish the stability of bacterial membranes and stimulate various immune functions (51, 52). In addition, reactive astrocytes express cell adhesion molecules such as ICAM-1 and VCAM-1 (50), inducible nitric oxide synthase (iNOS) with concomitant production of reactive nitrogen species (53, 54), and the PAMP-recognizing toll-like receptor 3 (TLR3), while other TLRs remain low to undetectable (55–58). Activation of TLR3 triggers a predominantly neuroprotective response, characterized by secretion of growth and differentiation mediators as well as pro- and anti-inflammatory cytokines (58).

Astrocytes also constitutively express low amounts of MHC-II and the adhesion molecules LFA-1 (CD11a) and ICAM-1 (CD54) (59). Stimulation with IFN-γ alone or in combination with TNF-α upregulates MHC-II, adhesion molecules, and co-stimulatory molecules B7-1 (CD80) and B7-2 (CD86) (60). Functional studies have shown that IFN-γ-treated murine astrocytes act as weak antigen-presenting cells, moderately activating $CD4^+$ and $CD8^+$ T cells. In contrast, cytokine-treated human astrocytes were not able to induce proliferation of encephalitogenic T cells, presumably because of lack of additional proliferation-inducing factors (59), suggesting interspecies differences in astrocytes. Furthermore, in the inflamed CNS, reactive astrocytes may contribute to B cell survival, maturation, and proliferation through production of B cell-activating factor of the TNF family (BAFF) (61–63). Other soluble factors secreted by astrocytes, such as IL-6 and IL-15, also support B cell survival (61). Finally, stimulation of astrocytes with cytokines diminishes their homeostatic and metabolic functions, resulting in impaired glutamate uptake, which may cause excitotoxicity, and in metabolic uncoupling from axons/neurons due to decreased release of lactate (23, 24, 64–66) (**Figure 1**).

Reactive astrocytes have recently been categorized according to their transcriptome profiles as "A1" or "A2," in analogy to the "M1" and "M2" phenotype categories for macrophages (67). A1-type astrocytes, for which complement component 3 is an identifying marker, are induced by inflammation (67, 68), are abundant in MS and neurodegenerative diseases, including Alzheimer's and Parkinson's disease, and secrete a neurotoxin that has not yet been identified (67). In contrast, A2-type

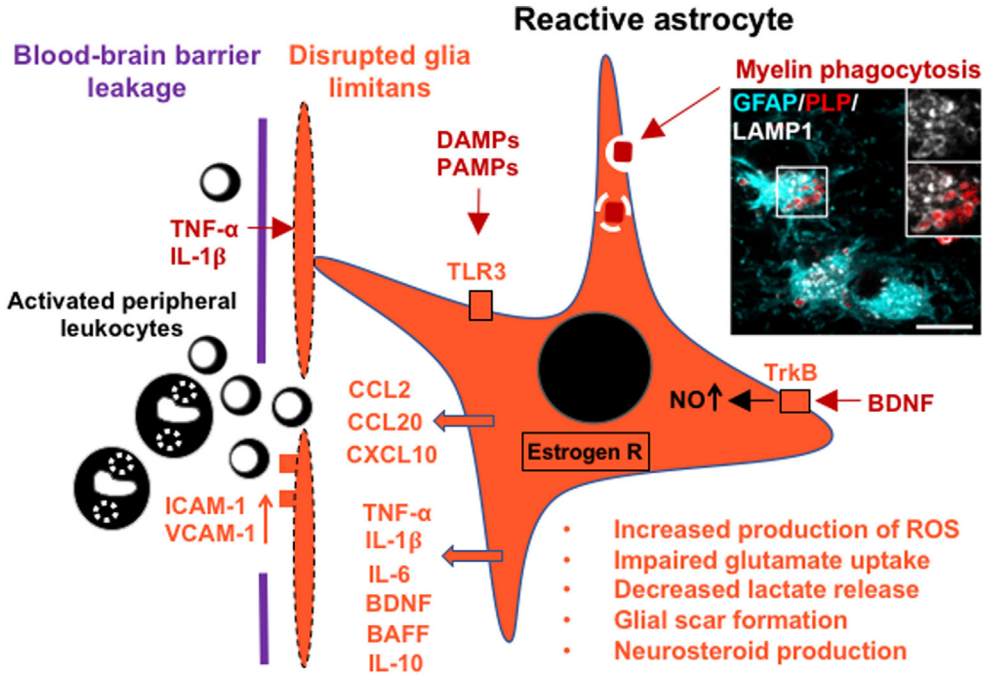

FIGURE 1 | Schematic of the glia limitans and a reactive astrocyte in multiple sclerosis (MS), indicating secretion of cytokines, recruitment of leukocytes across the blood–brain barrier, and upregulation of selected receptors that contribute to astrocyte responses and metabolic changes. Inset image shows a reactive hypertrophic astrocyte at the active rim of an MS lesion containing myelin debris within lysosomal ring structures [glial fibrillary acidic protein (GFAP), cyan; myelin proteolipid protein (PLP), red; lysosomal-associated membrane protein 1 (LAMP1), white]. Scale bar = 10 μm. The inset image was reproduced from Figure 1 of Ponath et al. (17) with the permission of Brain.

astrocytes are induced by ischemia and express neurotrophic factors (67). The concept of M1/M2 polarization is now considered an oversimplification and has been abandoned in favor of multiple, complex polarization states that do not necessarily align with a one-dimensional M1–M2 spectrum (69). Thus, while defining these phenotypes is an important step, reactive astrocytes may also exceed the A1–A2 dichotomy and assume a range of profiles with mixed A1 and A2 features (70). It has been proposed that although reactive astrocytes share common properties, they also display unique cellular and molecular features that are specific to different neuropathologies (70, 71). Moreover, distinct astrocytic phenotypes may coexist or develop sequentially during different phases of a pathological process: reactive astrocytes may first produce pro-inflammatory cytokines and reactive oxygen species in conjunction with hypertrophy and proliferation. In a second phase, astrocytes may promote anti-inflammatory and neuroregenerative functions through astrocyte-derived neurotrophic factors (72).

Thus, reactive astrocytes can mount powerful inflammatory responses that drive leukocyte recruitment to the CNS and thereby contribute to a successful defense against pathogens. Moreover, reactive astrocytes may change their response profiles over time, resulting in the secretion of anti-inflammatory cytokines and neurotrophins (72, 73). Astrocytosis can also aid in BBB repair and, depending on the severity of the injury, lead to the formation of glial scars that isolate the inflamed area, restrict damage and provide structural support (25).

THE REACTIVE ASTROCYTE IN MS LESION PATHOLOGY

Astrocytes are increasingly recognized as cells that critically contribute to the development of MS lesions. Previously, astrocytes were believed to react only at a late, post-inflammatory stage by forming a glial scar, but are now considered early and active players in lesion pathology (16, 17). In active lesions, astrocytes assume a hypertrophic morphology, characterized by massive enlargement of the cell soma and reduced process density (16). Typically, pronounced astroglial hypertrophy is indicative of substantial tissue injury and might be caused in MS lesions by oligodendrocyte loss and the resulting disruption of astrocyte–oligodendrocyte networks (16, 28). In addition, hypertrophic astrocytes may themselves sustain substantial damage that leads to retraction or loss of glia limitans from the basal lamina around blood vessels, presumably further increasing access of immune cells to the CNS (16) (**Figure 1**).

Reactive astrocytes are present in the active margins of demyelinating lesions and extend into adjacent, normal-appearing white matter (NAWM), suggesting that they are early contributors to lesion development (16, 17). This view is supported by the observation in murine experimental autoimmune encephalomyelitis (EAE), an inflammatory demyelinating model of MS, that astrocytes in nascent lesions become activated before significant immune cell infiltration into the parenchyma takes place (74–76). Furthermore, we have shown that hypertrophic astrocytes at

the leading edge of actively demyelinating MS lesions contain myelin debris (17). We have demonstrated that this myelin uptake induces astroglial NF-κB signaling and secretion of cell-recruiting chemokines. Therefore, we hypothesized that uptake of damaged myelin by astrocytes may be an early trigger for their activation, leading to astrocyte-mediated influx of leukocytes at the very beginning of lesion development (17) (**Figure 1**). Given that astrocytes in MS lesions express MHC class II and co-stimulatory molecules CD80 and CD86 (77, 78), it is tempting to speculate that myelin phagocytosis by astrocytes results in the presentation of myelin antigens to T cells. However, since stimulated human astrocytes in culture fail to induce, and even inhibit, proliferation of T cells (79), we consider it unlikely that astrocytes act as competent antigen-presenting cells in MS lesions.

In MS, hypertrophic astrocytes express chemokines and cell adhesion molecules associated with macrophage/microglia and lymphocyte recruitment into the parenchyma (80–82). Their functional relevance to leukocyte recruitment has been well documented in EAE. For example, in mice with a conditional, astrocyte-specific gene deletion of CCL2, induction of EAE resulted in a less severe disease course with fewer macrophage and T cell infiltrates, and less activation of astrocytes and microglia (83). Similarly, mice with a genetic deletion of all ICAM-1 isoforms showed marked attenuation of EAE, with minimal cellular infiltration and demyelination in the spinal cord (84). Conversely, astrocyte-mediated recruitment of microglia to demyelinating lesions is also of benefit, as demonstrated in a demyelination model using the oligodendrocyte toxin cuprizone, which does not disrupt the BBB or involve peripheral immune cell infiltration (85). Genetic ablation of astrocytes in mice treated with cuprizone prevented the recruitment of microglia cells to the site of demyelination, leading to delayed removal of myelin debris, impaired remyelination, and reduced proliferation of oligodendrocyte precursor cells (86). Thus, activated astrocytes are key regulators for the removal of damaged myelin, which is needed before remyelination can take place (86).

In addition, BAFF production by reactive astrocytes may contribute to the pathogenesis of MS by promoting B cell survival and proliferation in the CNS (61, 62). BAFF levels were shown to be increased in the CSF of MS patients compared to healthy controls (87). Moreover, BAFF mRNA was strongly upregulated in MS lesions, comparable to levels observed in lymphatic tissues, and BAFF was found to be expressed in reactive astrocytes, adjacent to inflammatory cells that expressed BAFF receptors (63). Given the continuous presence of antigen-experienced B cell clones in the CNS of MS patients (88) and the development of meningeal B cell follicles in progressive MS (89), astroglial production of BAFF may be a major factor to sustain these cells and to drive B cell-related pathology.

Reactive astrocytes likely contribute to tissue damage in MS through impaired glutamate handling and redox homeostasis. Glutamate concentrations were shown to be elevated in acute lesions of MS patients using *in vivo* MR spectroscopy (90). Moreover, a GWAS has linked specific risk variants associated with glutamate metabolism to increased cortical glutamate concentrations and poor disease outcomes in MS patients (91). In EAE, disease severity as well as oligodendrocyte and neuronal death were ameliorated through treatment with antagonists to the AMPA/Kainate or NMDA type of glutamate receptors (23, 24).

A recent study in a chronic progressive model of EAE has shown that astrocytes produce and are stimulated by the sphingolipid lactosylceramide (LacCer) (7). LacCer induces production of pro-inflammatory cytokines and iNOS in astrocytes and promotes pathology during experimental spinal cord injury (92). In EAE, LacCer was found to control the recruitment and activation of microglia and CNS-infiltrating monocytes by astrocytes. In addition, inhibition of LacCer synthesis suppressed CNS innate immunity and neurodegeneration. Finally, LacCer and the LacCer synthase β-1,4-galactosyltransferase 6 (B4GALT6) were detected in reactive astrocytes within MS lesions (7), suggesting that the B4GALT6-LacCer pathway is relevant to human disease.

Although reactive astrocytes drive inflammatory and neurotoxic responses in MS lesions, they may also dampen inflammation and promote neuroprotection and lesion repair. A factor produced by astrocytes and neurons in the normal CNS, which has CNS-trophic effects, is BDNF (93, 94). In EAE, astrocyte-specific deletion of BDNF resulted in a more severe clinical course with increased axonal loss (95). Moreover, in the cuprizone mouse model, enhanced BDNF production by astrocytes, induced by stimulation of metabotropic glutamate receptors, resulted in enhanced remyelination (96). However, a separate study demonstrated that signaling through the BDNF receptor TrkB in astrocytes leads to production of nitric oxide (NO) (97). EAE induced in mice with astrocyte-specific genetic deletion of TrkB had ameliorated disease severity, concomitant with reduced expression of astrocytic and lesional iNOS (97). These data indicate that BDNF released by astrocytes not only elicits neuroprotective effects in other cell types but also stimulates production and release of toxic NO in astrocytes themselves. In MS lesions, BDNF is primarily present in immune cells and reactive astrocytes (98), while the BDNF receptor TrkB was strongly upregulated in reactive astrocytes and in neurons in the immediate lesion vicinity (98). This suggests a possible dual protective and degenerative role for BDNF.

Astrocytes are susceptible to neurosteroids, such as estrogen and DHEA, which downregulate pro-inflammatory responses in reactive astrocytes (99–101). This mechanism plays a significant role in EAE where treatment of mice with an estrogen receptor-α (ERα) ligand substantially ameliorated clinical symptoms, inflammatory infiltrates, and axonal loss (102, 103). These beneficial effects were mediated entirely through ERα expressed by astrocytes, as they were abolished in EAE induced in mice with conditional, astrocyte-specific deletion of ERα (103). In MS lesions, ERα, aromatase, an enzyme involved in estrogen synthesis, and progesterone receptor were found to be upregulated in reactive astrocytes (104), suggesting that neurosteroid synthesis by reactive astrocytes as well as astrocytic responses to neurosteroids are part of an endogenous protective mechanism. On the other hand, a recent study found that the neurosteroids allopregnanolone and DHEA were substantially downregulated in EAE and in NAWM of autopsied MS tissue (105). Provided that astrocytes are the main steroidogenic cells in the brain (38), these

data may point toward impaired synthesis of both neurosteroids by astrocytes in MS.

In addition, TLR signaling may play a neuroprotective role in EAE and by extension, in MS, although this effect might not be astrocyte-specific. Systemic administration of the TLR3 agonist polyinosinic:polycytidylic acid (poly I:C) in EAE suppresses relapsing demyelination through induction of IFN-β and other immune regulatory effects (106). Furthermore, TLR4 knockout mice exhibited more severe EAE symptoms than wild-type mice, associated with increased priming of encephalitogenic Th17 cells (107). In MS lesions, TLR3 and 4 are expressed by microglia and astrocytes, where astroglial TLR expression is particularly prominent at later stages of inflammation, which may be instrumental in mitigating inflammation and promote tissue repair (56, 58).

Furthermore, following acute inflammation and demyelination, hypertrophic astrocytes eventually form a glial scar in the center of white matter lesions (25). While scars have been considered as barriers to tissue regeneration (16), they also provide beneficial features and contribute to recovery from CNS insults (25). For example, glial scars support demyelinated axons, help restore BBB function, and confine inflamed areas, preventing the spread of immune cells and toxic levels of extracellular ions, metabolites, or DAMPs into healthy tissues or areas of repair (16, 25).

Recent studies have implicated gut microbiota in immunological disorders including MS and its animal model, EAE (108, 109). The microbiome has emerged as a regulator of BBB integrity, where the absence of normal gut flora leads to disorganization of tight junctions in endothelial cells (110), and the production of short-chain fatty acids by bacteria corrects BBB dysfunction (111, 112). However, to date, astrocytes have not been found to mediate these effects.

SIGNALING PATHWAYS IN ASTROCYTES

Astrocyte reactivity is regulated by key canonical signaling cascades, among which the NF-κB pathway is pivotal for establishing neuroinflammation (113) (**Figure 2**). NF-κB is a master regulator of innate and adaptive immunity that controls cell survival, differentiation, and proliferation (114). Astrocytic NF-κB signaling is directly activated through stimulation with the pro-inflammatory cytokines TNF-α and IL-1β (113), through TLR signaling and various other agents including phagocytosed myelin, mitogens, and free radicals (17, 113, 115, 116). NF-κB signaling in astrocytes plays a critical role for initiating and maintaining inflammation in the CNS. Transgenic mice with astrocyte-specific inactivation of NF-κB display dramatic amelioration of tissue damage and clinical impairment following induction of EAE, spinal cord injury, or ischemic retinal injury compared to wild-type mice (117–119). Similarly, ablation of IL-17-induced Act1 signaling in astrocytes, which abolishes IL-17-mediated NF-κB activation, reduces the recruitment of lymphocytes and macrophages and markedly ameliorates disease severity in EAE (120).

Interestingly, microbial flora and its products have been shown to control NF-κB signaling through conversion of dietary tryptophan into agonists of aryl hydrocarbon receptor (AHR), which is highly expressed by astrocytes (121). AHR suppresses the classical activation pathways of NF-κB through competitive binding to the NF-κB subunit p65 (122). Induction of EAE in mice with astrocyte-specific genetic knockout of AHR [glial fibrillary acidic protein (GFAP)–AHR^{-}] resulted in increased expression of chemokines, cytokines and pro-inflammatory markers and an exacerbated disease course compared to wild type animals. Moreover, mice fed with a tryptophan-depleted diet exhibited a more severe EAE course, which could not be reversed by addition of tryptophan in GFAP–AHR^{-} mice. In MS, *AHR* expression was upregulated in active and chronic MS lesions and localized to GFAP^{+} astrocytes; however, this might not translate into AHR-dependent downregulation of astrocyte activation, because expression of the AHR transcriptional target *CYP1B1* was decreased in MS lesions and NAWM, suggesting that this pathway is defective in MS (121).

We have recently shown that an MS risk variant, rs7665090, which increases NF-κB signaling in lymphocytes (123), substantially affects astrocyte reactivity in cell culture and MS white matter lesions (81). Astrocytes derived from induced pluripotent stem cells, obtained from MS patients carrying the risk variant, showed increased NF-κB activation, chemokine and cell adhesion molecule expression, as well as impaired glutamate uptake and reduced lactate release. In addition, the risk variant was associated with significantly higher numbers of infiltrating lymphocytes in white matter MS lesions and with an increased lesion load on MRI in MS patients (81). Therefore, this NF-κB-relevant risk variant promotes pro-inflammatory changes in astrocytes that might help target aberrant immune responses to the CNS. This challenges the view that MS is mediated solely through dysregulation of lymphocytes and highlights the importance of astroglial NF-κB signaling for lesion formation (81) (**Figure 2**).

An important but less elucidated signaling cascade for regulation of astrocyte activation in MS is the STAT3 pathway (**Figure 2**). STAT3 activity is generally upregulated in response to CNS inflammation and damage (124). In astrocytes, STAT3 signaling is induced by both pro- and anti-inflammatory molecules, including IFN-γ and cytokines of the IL-6 family, that bind to the gp130 cell-surface receptor to induce STAT3 phosphorylation (124–127). STAT3 signaling in astrocytes plays a beneficial role in CNS inflammation, as demonstrated in mice with conditional, astrocyte-specific knockout of STAT3, where spinal cord injury lesions exhibited increased demyelination, contained more infiltrating dendritic cells, and had attenuated astrocyte hypertrophy and glial scar formation (128, 129). Similarly, in EAE, disease severity was exacerbated in mice with astrocyte-specific knockout of the STAT3-activating gp130 signal transducer, with larger areas of demyelination and increased infiltration of reactive T-lymphocytes (130). Moreover, activated astrocytes have been shown to provide neuronal protection *via* ERK (131) and/or STAT3 signaling during inflammation (132). This was demonstrated in an *in vivo* model of acute LPS/IFN-γ-induced neuroinflammation, where STAT3 and ERK signaling induced IL-6 production, which protected against neuronal apoptosis (133). Despite its importance as a neurotrophin in the CNS, IL-6 is also known to promote MS lesion development when produced in excess (134, 135). Specifically, IL-6 inhibits differentiation of naïve T cells into regulatory T cells and promotes their differentiation into

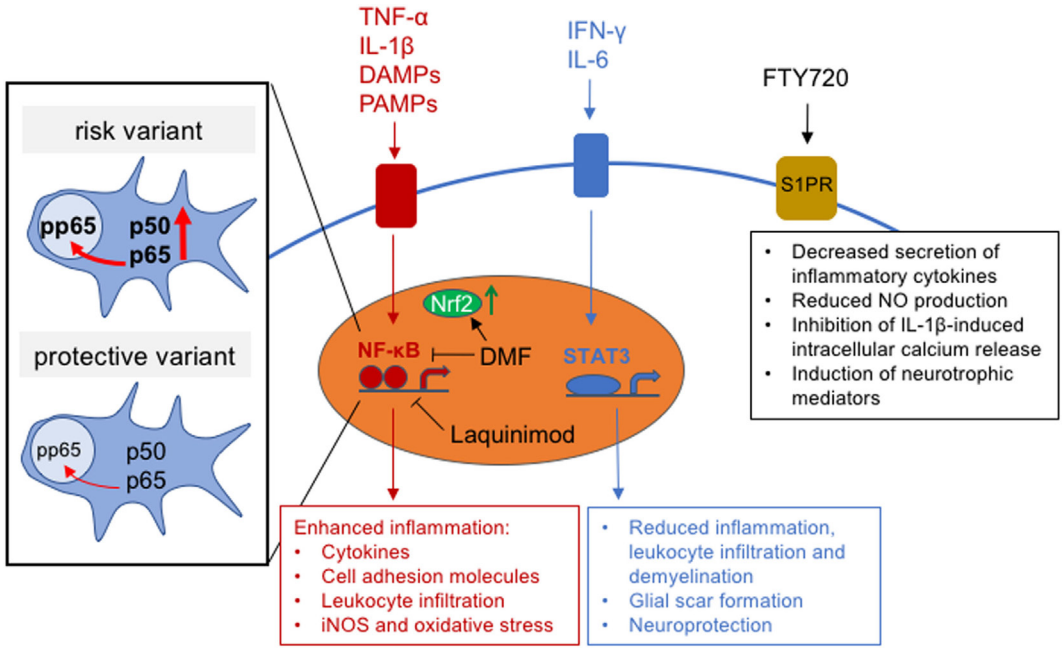

FIGURE 2 | Intracellular astrocytic signaling pathways and effects of multiple sclerosis (MS) treatments on signaling function. Inset shows schematic of the effect of MS risk variant, rs7665090, on NF-κB subunit expression and NF-κB signaling.

Th17 helper cells, which are considered to be major mediators of MS pathology (134). Thus, IL-6 levels above or below a certain threshold may determine its role as either a growth factor and activator of STAT3-mediated anti-inflammatory function, or as a suppressor of regulatory T cell differentiation and enhancer of pro-inflammatory helper T cell activity. Therefore, understanding the dynamics of IL-6 production in CNS lesions may help to predict the effectiveness of STAT3 signaling as a suppressor of lesion pathology.

DIRECT MODULATION OF REACTIVE ASTROCYTE ACTIVITY BY MS TREATMENTS

As discussed above, activated astrocytes play multiple pivotal roles during inflammation, including regulation of leukocyte trafficking, release of neurotoxic factors, confinement of inflammation, and promotion of neuroprotection and tissue repair. This makes astrocytes obvious therapeutic targets in MS. Ideally, such treatments would take into account the multi-functionality of astrocytes to block detrimental responses and/or enhance regenerative properties. Current MS therapies that are known to cross the BBB and modulate astrocyte function are laquinimod, which is currently being developed as an MS treatment, dimethyl fumarate (DMF; Tecfidera®) and fingolimod (FTY720; Gilenya®)/siponimod. In clinical studies, siponimod and laquinimod have shown a positive impact on progressive MS and brain atrophy, respectively. Since progressive MS is believed to be driven in part by chronic glial activation, these studies provide circumstantial evidence that astrocyte activation may contribute to progressive pathology. Below, we provide details of how each of these compounds impacts astrocytes.

Laquinimod

Laquinimod is a small quinolone derivative of the immunomodulatory compound linomide. Laquinimod was initially tested in RRMS, where it led to moderate effects on the reduction of relapse rates as a primary study endpoint. However, significant effects were observed on brain atrophy and disease progression (136). This led to a clinical trial of laquinimod in primary progressive MS (ARPEGGIO trial), which is still ongoing (137).

While the precise molecular targets of laquinimod are not well defined, recent data suggests that laquinimod activates genes associated with the transcription factor aryl hydrocarbon receptor (AHR, see above) (138). AHR target genes encode for drug-metabolizing enzymes and proteins controlling cell proliferation, differentiation, and apoptosis (139, 140). Additionally, cross talk between AHR and other signaling pathways, including epidermal growth factor receptor (EGFR) (141, 142), protein kinase A (PKA) (143, 144), and NF-κB signaling (144, 145), has been reported (146). Accordingly, AHR modulates the differentiation and function of many cell populations, several of which play an important role in neuroinflammation. In mouse EAE, laquinimod exerts effects on the peripheral immune system, where it downregulates pro-inflammatory T cell responses (147, 148), and on CNS cells. Genetic deletion of AHR in the immune system fully abrogated the treatment effect of laquinimod on

EAE, while deletion of AHR in the CNS partially abrogated this effect (138). In a separate study, laquinimod markedly reduced NF-κB signaling and pro-inflammatory activation of astrocytes, but not of microglia *in vitro* (**Figure 2**). In the cuprizone model of demyelination, laquinimod prevented demyelination, microglial activation, T cell infiltration, and axonal transection; this effect was attributed to *in vivo* attenuation of NF-κB signaling in astrocytes (149). Laquinimod exhibits additional modes of action including neuroprotection, as demonstrated in EAE, where conditional deletion of BDNF in myeloid and T cells partially abrogated the beneficial effect of laquinimod. Similarly, laquinimod treatment of MS patients was found to increase expression of BDNF in serum (150). Taken together, current data suggests that laquinimod exerts effects on multiple cell types during CNS inflammation. A key mechanism mediated by laquinimod is the downregulation of pro-inflammatory responses in astrocytes. Thus, the beneficial effects of laquinimod on brain atrophy and disability progression in MS patients may at least partially be explained by its direct effect on astrocytes.

Dimethyl Fumarate

Dimethyl fumarate is the methyl ester of fumaric acid and was FDA-approved for the treatment of relapsing forms of MS in 2013. In placebo-controlled clinical trials, Tecfidera® reduced the relapse rate in MS patients by approximately 50% and disability progression by 38% in one trial but not in a separate, parallel trial (151, 152). The effect of Tecfidera® on SPMS is unclear, as a phase III clinical trial was initiated but terminated early due to restructuring of the drug's manufacturer, Biogen (153). DMF activates the Nrf2 transcription factor, which targets antioxidant response element (ARE) genes coding for antioxidant enzymes that reduce oxidative stress (154). DMF induces Nrf2 through glutathione depletion and direct binding to the Nrf2 repressor Kelch-like ECH-associated protein 1 (KEAP1) (155–158). Moreover, DMF acts as a potent inhibitor of NF-κB signaling (159) and has been shown to modify DMF-sensitive cysteine residues in human T cells, which inhibits T cell activation (160) (**Figure 2**).

In the peripheral immune system, DMF reduces lymphocyte counts, in particular cytotoxic and effector T cells, and inhibits activation of antigen-presenting cells (161). In the CNS, a major effect of DMF is the upregulation of Nrf2 in astrocytes, which is protective against oxidative injury *via* upregulation of oxidative stress-induced growth inhibitor 1 (162). This effect might ameliorate astrocytic damage in active lesions, including the retraction of perivascular astrocyte end feet along basal lamina (16), to reduce leakage across the BBB and the cortical surface (163). DMF also inhibits secretion of pro-inflammatory cytokines and chemokines by astrocytes and microglial cells, independent of changes in antioxidant gene expression (164). Therefore, in addition to its effect on the peripheral immune system, DMF has a direct impact on the CNS that involves protective and anti-inflammatory effects on astrocytes.

Fingolimod and Siponimod

FTY720/fingolimod(2-amino-2[2-(4-octylphenyl)ethyl]propane-1,3-diol hydrochloride; Gilenya®) is a non-specific sphingosine-I phosphate (S1P) modulator. In clinical trials with RRMS patients, FTY720 reduced the annualized relapse rate by 48–55% and decreased risk of disability progression by 28% in one study, while having no significant effect on disability in another (165, 166). With regards to primary progressive MS, a recent trial demonstrated that FTY720 had no beneficial effects on disability progression or whole-brain atrophy (167). However, Siponimod, a selective modulator of the $S1P_1$ and $S1P_5$ receptors, did slow disability progression in SPMS in a phase III clinical trial (168).

The main effect of FTY720 on the peripheral immune system is the internalization and degradation of the S1P receptor on lymphocytes, which results in impaired responses to the S1P gradient in lymph nodes and prevents lymphocyte egress (169, 170). In the CNS, S1P receptors play a number of roles in brain cell function, including astrocyte proliferation and migration (171, 172), oligodendrocyte differentiation and survival (173, 174), and neurite outgrowth and neurogenesis (175–177). The mechanism most relevant to MS and its animal model, EAE, involves $S1P_1$ receptor signaling in astrocytes, which has been demonstrated in conditional null mouse mutants lacking $S1P_1$ in astrocytes. When induced to develop EAE, these mice showed a substantial reduction in disease severity, which was not further affected through additional FTY720 treatment, suggesting that the main effect of FTY720 in EAE involves modulation of astrocyte function but not the arrest of lymphocytes in lymph nodes (178). In contrast, conditional deletion of $S1P_1$ in neuronal cell lineages had no impact on EAE severity or the efficacy of FTY720 to suppress EAE. Astrocytes mainly express $S1P_1$ and $S1P_3$ as well as other subtypes at low levels (171, 179). Expression of both receptors is markedly increased in reactive astrocytes in active and chronic MS lesions. Moreover, treatment of cultured human astrocytes with FTY720 limits secretion of inflammatory cytokines (180), reduces NO production (181), induces neurotrophic mediators, and inhibits TNF-α-induced inflammatory gene expression (182). Modulation of astrocytic intracellular pathway function induced by FTY720 includes enhanced expression of calcium-regulating proteins and inhibition of calcium release induced by the pro-inflammatory mediator IL-1β (183) (**Figure 2**). This data implicates $S1P_1$ signaling in astrocytes as a major contributor to the pathogenesis of EAE and as the main therapeutic target of FTY720 (184). Thus, the efficacy of Siponimod, a modulator of the $S1P_1$ and $S1P_5$ receptors, in slowing disability accumulation in SPMS may be mediated through its direct effect on astrocytes.

Other MS therapies, such as teriflunomide (Aubagio®) (185, 186) and IFN-β (Avonex®, Betaseron®, Rebif®), have been shown to inhibit astroglial immune responses, the latter by inducing astroglial expression of AHR (121). However, teriflunomide and IFN-β have limited or no BBB penetrance (187, 188), making it unlikely that these drugs exert continuous, direct effects on CNS-resident cells.

CONCLUSION

Astrocytes play an instrumental role in the formation of MS lesions through a multitude of functional changes associated

with their activation. Astrocytes are early responders in nascent white matter lesions, are the main recruiters of lymphocytes, and act themselves as immunocompetent cells that contribute to innate immunity. Moreover, astrocytes not only can adopt a neurotoxic phenotype, but also confine inflammation through scar formation and can promote neuroprotection and tissue repair. Astrocytic dysfunction associated with a genetic MS risk variant further suggests that astrocyte-mediated processes are causative in lesion pathology. Thus, while MS is driven by dysfunction of the peripheral immune system, CNS cells such as astrocytes may contribute to MS pathology by targeting dysregulated immune responses to the CNS. Finally, MS medications that impact astrocytes have shown efficacy in both relapsing–remitting and phase III clinical trials of progressive MS, providing further circumstantial evidence that activation of astrocytes contributes to both pathologies.

AUTHOR CONTRIBUTIONS

GP, CP and DP wrote the manuscript.

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Adenosine A2A Receptor Signaling in the Immunopathogenesis of Experimental Autoimmune Encephalomyelitis

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Our increasing appreciation of adenosine as an endogenous signaling molecule that terminates inflammation has generated excitement regarding the potential to target adenosine receptors (ARs) in the treatment of multiple sclerosis (MS), a disease of chronic neuroinflammation. Of the four G protein-coupled ARs, A2ARs are the principal mediator of adenosine's anti-inflammatory effects and accordingly, there is a growing body of evidence surrounding the role of A2ARs in experimental autoimmune encephalomyelitis (EAE), the dominant animal model of MS. Such evidence points to a complex, often paradoxical role for A2ARs in the immunopathogenesis of EAE, where they have the ability to both exacerbate and alleviate disease severity. This review seeks to interpret these paradoxical findings and evaluate the therapeutic promise of A2ARs. In essence, the complexities of A2AR signaling arise from two properties. Firstly, A2AR signaling downregulates the inflammatory potential of TH lymphocytes whilst simultaneously facilitating the recruitment of these cells into the CNS. Secondly, A2AR expression by myeloid cells – infiltrating macrophages and CNS-resident microglia – has the capacity to promote both tissue injury and repair in chronic neuroinflammation. Consequently, the therapeutic potential of targeting A2ARs is greatly undermined by the risk of collateral tissue damage in the periphery and/or CNS.

Keywords: adenosine, adenosine 2A receptor, experimental autoimmune encephalomyelitis, multiple sclerosis, neuroinflammation, microglia

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INTRODUCTION

Multiple sclerosis (MS) is the most common chronic neuroinflammatory disease in the Western World, affecting ~2.5 million people worldwide, typically in the third and fourth decades of life (1). The equipoise between genetic and environmental factors is undoubtedly central to the etiology of MS yet despite years of research, the precise cause of MS remains elusive. Inflammation, demyelination, reactive gliosis, and neuroaxonal degeneration characterize CNS lesions observed in MS patients, and the heterogeneous spatiotemporal dissemination of these lesions is reflected by the heterogeneous clinical presentation of MS. This typically includes some combination of somatosensory and visual defects, impairments in pyramidal-motor control, fatigue, pain, and cognitive deficits.

The immunopathogenesis of MS is characterized as a T cell-mediated autoimmune response against myelin self-antigen, which provokes the migration of immune cells across the blood–brain

barrier and blood–CSF barrier (2). Within the CNS, macrophages and T cells (both $CD4^+$ and $CD8^+$) dominate the inflammatory infiltrate. EAE, the principal animal model of MS, has been fundamental in investigating the immunopathogenic mechanisms underlying MS. This is because it can recapitulate the cardinal pathological features of MS observed in patients, namely inflammation, demyelination, axonal loss and gliosis (3). Experimentally, it is induced by immunizing animals with myelin derived proteins—typically myelin oligodendrocyte glycoprotein (MOG) in mice—which results in the generation of primed $CD4^+$ T helper 1 (T_H1) and T_H17 cells, which in turn drive EAE pathogenesis. EAE is able to accurately recapitulate the early, *inflammatory* phase of MS, during which a degree of remyelination is possible. However, in the second phase of MS, axonal degeneration commences and remyelination becomes increasingly difficult. This *neurodegenerative* phase is less accurately recapitulated by EAE, which is, after all, immunological in nature. Accordingly, a different set of mechanisms must be considered to explain the distinct, neurodegenerative component of MS.

A handful of immunomodulatory agents have had success in managing relapsing–remitting MS, the most common clinical form of MS. First generation therapies such as IFN-β and glatiramer acetate reduce both the frequency and severity of relapse and have good safety records, but they do not substantially halt disease progression (4). Among the newly developed monoclonal antibody (mAb) therapies, the most notable is alemtuzumab, which is significantly more efficacious in reducing relapse rates than first generation therapies and unprecedentedly, is able to improve long-term disability outcomes; however, concerns regarding the safety profile of alemtuzumab have been raised (5). Furthermore, only one drug—ocrelizumab—has been approved specifically for primary progressive MS, and no treatments have been approved specifically for secondary-progressive MS. Thus, given the magnitude of the disease burden, MS remains a major clinical challenge with scope for novel therapeutic targets and EAE remains instrumental in addressing this challenge.

PURINERGIC SIGNALING IN INFLAMMATION

Cellular stress or apoptosis induces the release of ATP into the extracellular space, promoting rapid inflammation by activating ATP receptors of which there are two subtypes, inotropic P2X receptors and metabotropic P2Y receptors (6). Both P2XRs and P2YRs amplify T cell receptor as well as innate immune signaling. Indeed, the potent ability of ATP to promote inflammasome activation in macrophages and dendritic cells renders it an important "damage-associated molecular pattern" in the acute inflammatory response to cellular damage and destruction (7–9).

The accumulation of extracellular ATP described above characterizes the acute phase of purinergic signaling, which lasts minutes to hours (6). In the subacute phase of purinergic signaling, lasting days to weeks, the extracellular ratio of ATP/adenosine declines. Correspondingly, there is a reduction in ATP signaling concomitant to an increase in the activation of P1 ARs, which serves to restrict the degree and duration of inflammation. Ordinarily, adenosine that accumulates in the extracellular environment is rapidly taken up *via* nucleoside transporters into neighboring cells, where adenosine is metabolized either by adenosine kinase to form AMP or by adenosine deaminase to form inosine; however, under inflammatory conditions, adenosine removal cannot keep pace with its generation. This increase in extracellular adenosine (**Figure 1A**), from basal nanomolar concentrations to ~10–50 μM, has potent and well-documented anti-inflammatory effects *via* one or more of four G protein-coupled ARs, denoted A1, A2A (**Figure 1B**), A2B, and A3. Finally, in the chronic phase of purinergic signaling, the low extracellular ratio of ATP/adenosine is associated with wound healing and can, on occasion, lead to pathological tissue remodeling.

From this temporal analysis, established recently by Cekic and Linden (6), it follows that that the accumulation of extracellular adenosine and activation of P1 ARs increase over time, bearing particular relevance to chronic neuroinflammatory conditions such as in EAE.

A2ARs are widely expressed in the CNS and among the key peripheral immune cells implicated in EAE (**Table 1**); furthermore, evidence from EAE studies and a range of other inflammatory conditions suggest that A2ARs are the prime mediator of adenosine's anti-inflammatory effects (11–13). Correspondingly, interest in the role of A2AR signaling in the immunopathogenesis of EAE has blossomed and it has been suggested that A2ARs may offer a novel therapeutic target for MS.

Of course, an evaluation of the therapeutic potential of A2ARs requires both an overview of A2A receptor regulation and an appreciation of the complex role of A2AR signaling in the progression of EAE. Underlying these complex effects are, firstly, the paradoxical effects of A2AR signaling in the recruitment of lymphocytes to the CNS and, secondly, the paradoxical effects of A2AR signaling in both infiltrating macrophages and CNS-resident microglia, during chronic neuroinflammation. Importantly, this interpretation must be evaluated against the limitations of EAE as an animal model of MS, with an emphasis on those limitations that apply to A2AR signaling in particular.

A2A RECEPTOR REGULATION

The molecular basis of A2A receptor regulation was investigated in pioneering studies into A2AR gene structures, which were shown to be highly conserved across mice, rats and humans (10, 25). The A2AR gene is composed of multiple exons that encode alternative transcripts, which are initiated from at least four independent promoters. Of the transcripts identified to date, they share identical coding regions and a common 3′ untranslated region (UTR) but distinct 5′ UTRs; thus, the function of these distinct 5′ UTRs is of particular interest in elucidating A2AR regulatory mechanisms. 5′ UTRs corresponding to the P2 and P3 A2AR promoters appear to suppress A2AR expression at the translational level while the regulatory function of 5′ UTRs that correspond to the P1A and P1B A2AR promoters is unclear. Moreover, transgenic studies in rats suggest that P1A, P2, and P3 promoters are responsible for A2AR expression in the CNS (26), which raises the possibility that the P1B promoter might regulate peripheral A2AR expression. Looking forward, it will be important to identify the DNA elements underlying the

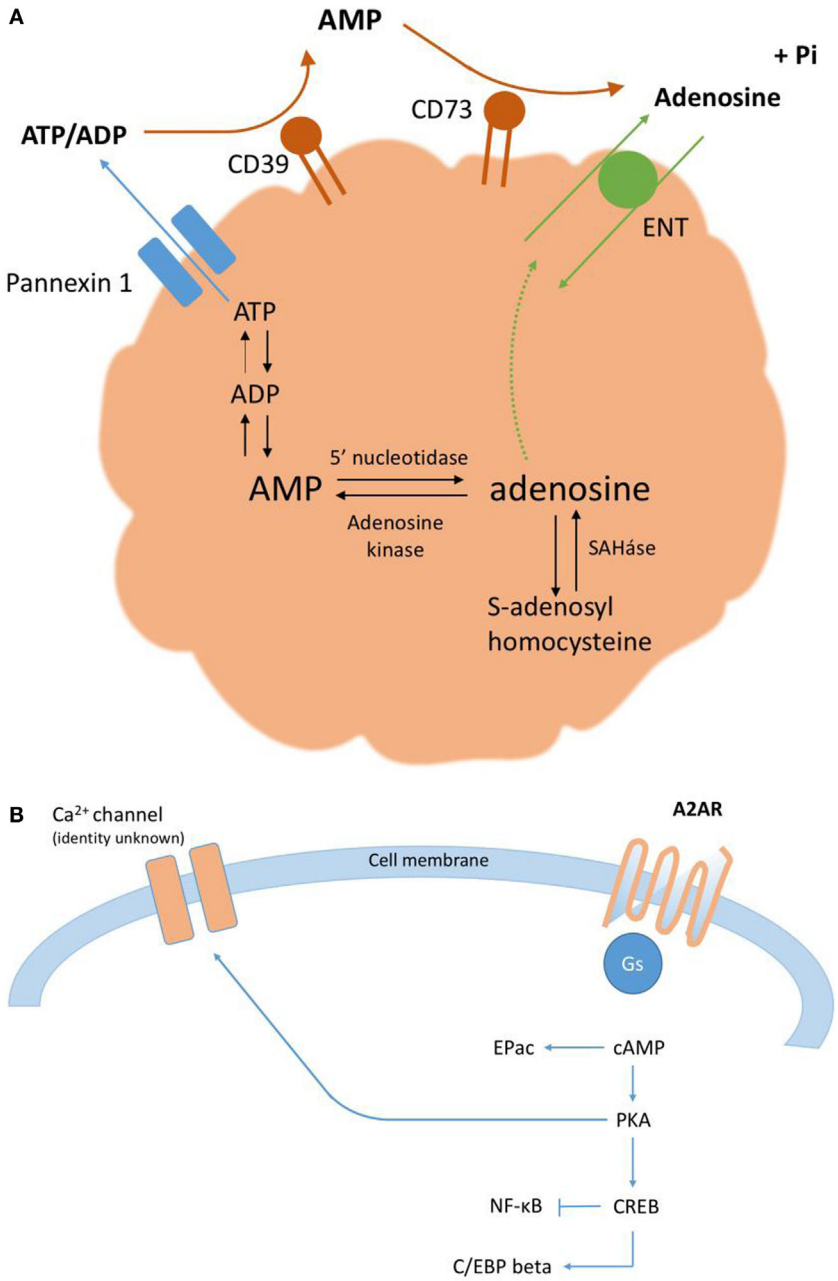

FIGURE 1 | **(A)** Extracellular adenosine accumulates *via* the breakdown of ATP, both intracellularly and extracellularly. **(B)** A2ARs signal predominantly *via* the adenylate cyclase-cAMP-protein kinase A (PKA) canonical pathway (10). PKA phosphorylates the transcription factor CREB on serine residue 133; activated CREB can affect gene expression directly, *via* specific promoters, or indirectly, *via* an important cofactor, CBP. cAMP can also signal directly *via* the exchange factor Epac.

intense expression of A2ARs in the striatum, which have not been recapitulated by the transgenic approach employed in these experiments.

In states of both inflammation and chronic neurodegenerative disease, changes in A2AR expression are well documented. In both murine and human macrophages, lipopolysaccharide induces an increase in A2AR mRNA expression in an NF-κB-dependent manner (27). By contrast, in Huntington's disease, the mutant Huntingtin gene exerts transcriptional suppression of striatal A2ARs *via* CREB inhibition (28). However, changes in A2AR expression in MS, which is characterized by both chronic neuroinflammation and neurodegeneration, and exhibits subtler genetic mechanisms, remain poorly characterized.

Thus, further characterization of the A2AR gene is necessary if we are to understand the molecular basis of how A2A receptors are physiologically regulated and indeed how they can be pharmacologically manipulated under pathophysiological conditions for therapeutic purposes.

TABLE 1 | The expression and function of A2ARs in the CNS and peripheral immune system.

Region of A2AR expression	Functional effects of increased extracellular adenosine/ATP
Central nervous system	
– Striatum	Postsynaptic reciprocal inhibitory interactions with D2 receptor signaling in striatopallidal medium spiny neurons (MSNs) involved in locomotor control (14)
	Presynaptic facilitation of glutamate release from cortico-striatal glutamatergic terminals in contact with striatonigral MSNs involved in locomotor control (14)
– Prefrontal cortex	Modulates acetylcholine release and inhibits cortical and behavioral arousal (15)
– Hippocampus	At the cellular level, facilitates excitatory glutamatergic Schaffer collateral synapses to CA1 pyramidal cells (16)
	Behaviorally, optogenetic stimulation of A2AR signaling pathways induces an impairment of spatial memory (17)
Peripheral immune system	
– $CD4^+$ [T helper 1 (T_H1) cells]	Anti-inflammatory—inhibits production of a range of cytokines inc. IL-2, TNF-α, and IFN-γ but has little effect on IL-4 and IL-5 production (18, 19)
– $CD4^+$ (T_H17 cells)	Anti-inflammatory—little effect on cytokine production but inhibits development of T_H17 cells (20)
– Invariant natural killer cells	Anti-inflammatory—inhibits IFN-γ production (20)
– $CD8^+$	Anti-inflammatory—mild impairment of proliferation but significant inhibition of IFN-γ and associated cytotoxicity (21)
– T_{reg}	Anti-inflammatory—encourages T_{reg} development in naïve T cells. Furthermore, expression of CD39 and CD73 on T_{regs} facilitates increase in adenosine availability (20)
– Macrophages and dendritic cells	Anti-inflammatory—reduces capacity to induce T_H1 polarization in naïve $CD4^+$ T cells, reduces production of pro-inflammatory TNF-α and IL-12, and enhances release of anti-inflammatory IL-10 (20)
Central nervous system immune system	
– Microglia	Enables a response to CNS inflammation by triggering process retraction and amoeboid morphology (22)
	Anti-inflammatory—inhibits microglial activation, which is implicated in release of both pro-inflammatory cytokines and reactive oxygen species (23)
	Pro-inflammatory—may facilitate production of inflammatory mediators such as nitric oxide and inhibit remyelination (24)
– Choroid Plexus Epithelium	Pro-inflammatory—may facilitate the transmigration of lymphocytes into the CNS (23)

A2AR SIGNALING HAS PARADOXICAL EFFECTS ON LYMPHOCYTE RECRUITMENT TO THE CNS DURING EAE

The most direct evidence of a role for A2ARs in the immunopathogenesis of MS comes from EAE animal studies, in which it has been shown repeatedly that knocking out A2ARs exacerbates the severity of EAE, as evidenced by greater motor paralysis, more infiltrating $CD4^+$ T lymphocytes in the CNS and more demyelination in A2AR KO ($A2AR^{-/-}$) mice in comparison with WT mice (23, 29). A possible confound in $A2AR^{-/-}$ mice induced with EAE is the loss of neuronal A2AR expression in the dorsal striatum and so the motor paralysis observed in EAE $A2AR^{-/-}$ mice may in part be attributable to impaired striatal motor control, in addition to the expected loss of A2AR signaling in the immune system. Importantly, therefore, the results of genetic knockout studies have been validated by pharmacological studies in lymphocytes isolated from MS patients (18). Stimulating A2ARs with the A2AR agonist CGS21680 significantly inhibits lymphocyte proliferation, VLA-4 expression and the release of a range of pro-inflammatory cytokines, including TNF-α, IFN-γ, IL-6, IL-1-β, and IL-17, all of which have been shown to contribute to MS progression (19, 30).

However, in direct contrast to these observations, it has been repeatedly shown that pharmacologically antagonizing A2ARs with SCH58261 confers protection against the induction of EAE in WT mice (23, 31). This contradiction was investigated in a series of elegant adoptive transfer experiments using the radiation bone marrow chimera model system (23). Adoptively transferring $A2AR^{-/-}$ $CD4^+$ T lymphocytes into $A2AR^{+/+}$ tcr-deficient mice induced an EAE pathology more severe than when WT $CD4^+$ T lymphocytes were adoptively transferred into $A2AR^{+/+}$ tcr-deficient mice and even, crucially, more severe than when $A2AR^{-/-}$ $CD4^+$ T lymphocytes were adoptively transferred into the $A2AR^{-/-}$ phenotype. Furthermore, the transfer of $A2AR^{+/+}$ lymphocytes into $A2AR^{-/-}$ mice did not induce EAE and importantly, neither $FoxP3^+$ immunostaining nor $T_{effector}$ suppression assays suggested any confounding alterations in T_{reg} frequency and/or functionality in $A2AR^{-/-}$ mice. These findings suggest that A2AR expression in T_H lymphocytes is essential for limiting the severity of the inflammatory response in EAE, while A2AR expression on radiation-resistant, non-hematopoietic cells promotes severe EAE. Thus, while knocking out A2ARs increases susceptibility to developing severe EAE due to the increased pro-inflammatory nature of $A2AR^{-/-}$ lymphocytes, it appears that SCH58261's blockade of A2ARs is protective *via* its action on non-hematopoietic cells.

The non-hematopoietic cells of interest are the choroid plexus epithelium (CPE), in which fluorescence *in situ* hybridization (FISH) studies reveal a high degree of A2AR and CD73 mRNA expression. Furthermore, this structure is an established CNS entry point for immune cells in MS (32–34). However, FISH does not functionally demonstrate the capacity of CPE A2AR signaling to mediate lymphocyte transmigration in EAE.

Another study compared the effects of introducing an A2AR agonist, CGS21680, at different time points following MOG immunization in an attempt to investigate how the role of A2AR signaling changes throughout the course of EAE (35). Introducing

CGS21680 on the day of MOG immunization reduced the severity of EAE and consistent with previous findings, adoptive transfer experiments demonstrated the mechanism of such protection to be the downregulated inflammatory potential of A2AR-expressing lymphocytes. However, introducing this CGS21680 12 days postimmunization (i.e., at the peak of the disease) exacerbated the severity of EAE in comparison with vehicle-treated mice. One possible limitation of this study is that these effects were not shown to be reversible with a selective A2AR antagonist. Indeed, given that A2AR–A2BR heterodimerization has been documented (36), and knocking out A2BRs also exacerbates the severity of EAE (37), it is possible that CGS21680 exerted confounding non-specific effects on A2BRs.

While Ingwersen et al. do not offer an interpretation of this paradox, it is plausible that the opposing effects of A2ARs signaling in T_H lymphocytes vs non-hematopoietic cells may account for these remarkable observations, especially since in the disease course of EAE, the peripheral activation of T_H cells occurs primarily in the first week post-immunization and by day 12, immune cells have begun to infiltrate the CNS in increasing numbers (5). To substantiate this correlation between the time-dependent effects of CGS21680 and the A2AR-sensitive progression of EAE, it would be necessary to investigate whether the stage of EAE at which an A2AR agonist is introduced affects the infiltration of adoptively transferred lymphocytes into the CSF.

A2AR SIGNALING IN T_{reg} LYMPHOCYTES IN EAE

The significance of A2AR signaling in lymphocytes in particular is further supported by its ability to shape the immune response *via* T_{reg} control. This is because FoxP3$^+$ T_{reg} cells are unique among T cells in their surface expression of both CD39 and CD73 ectoenzymes and thus, in their ability to generate pericellular adenosine from extracellular ATP and ADP (38). This, in addition to the marked expression of A2ARs on T effector cells, places A2AR activation at the center of T_{reg}-mediated immunosuppression.

Indeed, the augmentation of T_{reg}-mediated immunosuppression can alleviate variants of EAE (39). For example, in both C57Bl/6 and SJL recipient mouse strains, which model chronic and relapsing–remitting forms of MS, respectively, it has been shown that passively transferring peripheral CD4$^+$ CD25$^+$ T cells from mice with EAE suppresses the development of chronic EAE in recipient mice (40). Similarly, passively transferring a small number of CNS-derived T_{reg} cells isolated from mice in the recovery phase of EAE considerably alleviated MOG-induced EAE in recipients (41). Interestingly, passively transferring an identical number of CD4$^+$ CD25$^+$ T cells from lymph nodes did not alleviate EAE in recipients. The greater capacity of CNS-derived T_{reg} cells to downgrade inflammation in comparison with peripheral T_{reg} cells highlights the importance of antigen specificity in T_{reg}-mediated immunosuppression in classical MOG-induced EAE. Furthermore, in the Tg MBP/Rag$^{-/-}$ EAE mouse model—in which transgenic mice expressing a TCR against myelin basic protein are crossed to mice of a recombination-activation gene 1—deficient background—T_{reg} cells are central in the resistance to EAE development in Tg MBP/Rag$^{+/+}$ mice (42). Further to this, it was shown that adoptively transferring CD4$^+$ CD25$^+$ T cells to Tg MBP/Rag$^{-/-}$ mice engenders resistance to spontaneous EAE development. In addition to these findings from EAE studies, an increasing amount of evidence supports a role for T_{reg} cells in MS in which CD4$^+$ CD25^{high} T_{reg} cells may be functionally impaired in their maturation and emigration from the thymus (43–46).

In summary, A2AR signaling is of central importance in T_{reg}-mediated immunosuppression and T_{reg} cells have been demonstrated to mitigate against the development and progression of a range of EAE models. A direct investigation into the role of T_{reg} A2AR signaling in EAE, perhaps involving the conditional genetic deletion of these receptors in CD4$^+$ CD25$^+$ T cells, is an obvious next step in understanding lymphocytic A2AR signaling in the context of EAE.

A2AR SIGNALING POTENTLY REGULATES MONOCYTE/MACROPHAGE-DERIVED TNF-α, WHICH HAS CONTRASTING EFFECTS IN EAE AND MS

A2ARs are highly expressed on infiltrating macrophages, which predominate in lesions in both EAE and MS, and the numbers of which correlate to tissue damage (6). In murine monocytes, knocking out A2ARs produces a significant upregulation of TNF-α production (47) while stimulating A2ARs with CGS21680 produces a significant downregulation of TNF-α production (48). These findings are validated by clinical observations of elevated CSF levels of TNF-α (49) and reduced plasma levels of cAMP in MS patients in comparison with control subjects (50). Furthermore, administering antibodies that neutralize TNF-α has been shown to abrogate EAE development (51) and the overexpression of TNF-α in transgenic mice results in lesions of demyelination mirroring those observed in MS patients (52). This suggests a role for TNF-α in potentiating demyelination. Unexpectedly, however, the TNF-α receptor blocker Lenercept was found to dose dependently increase the frequency of relapse in MS patients in phase II clinical trials (53), suggesting that TNF-α plays a more complex role in MS. Further investigations in oligodendroglia found that TNF receptor I mediates nerve demyelination whereas TNF receptor II is essential to nerve remyelination. Indeed, the expression of TNF receptor II alone was sufficient to restore oligodendrocyte regeneration in TNF-α$^{-/-}$ mice (54). Thus, it is difficult to envisage the A2AR-mediated modulation of TNF-α release as a promising therapeutic avenue given that this cytokine can promote both the progression and regression of MS depending on the TNF receptor subtype it activates.

A2ARs also upregulate the release of IL-10, an anti-inflammatory cytokine that acts directly on CD4$^+$ T cells, inhibiting proliferation as well as the release of TNF-α, IL-2, IFN-γ, IL-4, and IL-5. It has been shown that IL-10 levels are reduced in MS patients and restoring them back to physiological levels may be one of the elusive therapeutic mechanisms of IFN-β-1b (55). Thus, inducing IL-10 release *via* A2AR agonism could compliment

IFN-β-1b treatment. However, in light of the contrasting effects of TNF-α in MS, directly administrating IL-10 may be a more promising therapeutic avenue than A2AR modulation.

Interestingly, the control of monocyte/macrophage-derived TNF-α by A2AR signaling has elucidated the importance of oxygen availability in the recruitment of the adenosine signaling system. It has been shown repeatedly that A2ARs are instrumental in the downregulation of TNF-α in murine macrophages in response to hypoxia (56, 57), a switch that involves the induction of HIF-1α by TLR4 activation and post-transcriptional stabilization of HIF-1α by A2AR signaling. The link between HIF-1α and A2ARs and more generally, the increased adenosine release by cells in hypoxic environments, suggests oxygen availability could be a fundamental trigger in recruiting adenosine signaling. Indeed, in light of recent findings, this may have relevance to the immunopathogenesis of EAE. Using novel fiber-optic PO_2 sensors, oxygenation in cortical and cerebellar gray matter was quantified in awake, unrestrained mice with MOG-induced EAE (58). Both cortical gray matter and cerebellar gray matter were hypoxic, and cortical gray matter hypoxia correlated with behavioral deficits. Of course, considering the contrasting effects of hypoxia-related inflammatory mediators such as monocyte/macrophage-derived TNF-α in white matter lesions, it is unclear whether A2AR signaling sustains or alleviates gray matter inflammation in EAE. Thus, further characterization of A2AR signaling in the context of hypoxia-related gray matter inflammation is warranted.

MICROGLIAL A2AR SIGNALING HAS CONTRASTING EFFECTS IN EAE

Similar to infiltrating macrophages, microglia also have the ability to promote both tissue injury and repair (59, 60), and A2ARs appear capable of facilitating both of these contrasting effects.

A number of studies have linked microglial activation in EAE to demyelination, the release of pro-inflammatory cytokines and the production of reactive oxygen species. Consistent with these findings and the protective, anti-inflammatory effects of A2ARs observed elsewhere, it was recently shown that the more severe EAE phenotype in $A2AR^{-/-}$ mice exhibited more $Iba1^+$ cells [Iba1 is a specific marker of microglial activation (61)] than WT mice in post-mortem sections (23). However, this observation does not distinguish between the enhanced microglial activation resulting from the increase in TNF-α release by infiltrating macrophages and lymphocytes that now lack A2ARs, and the enhanced microglial activation resulting from the absence of microglial A2AR signaling. Moreover, this finding is potentially confounded by developmental changes in A1R expression in $A2AR^{-/-}$ mice, given that A1Rs are expressed in microglia and especially since A1R KO studies have implicated these receptors in EAE progression (62).

By contrast, evidence from cultured microglial cells indicates microglial A2AR signaling has the capacity to exacerbate EAE. CGS21680 concentration dependently potentiates LPS-induced nitric oxide (NO) and NO synthase-II expression, both of which characterize the microglial inflammatory response (24) and indeed, A2AR blockade curtails LPS-induced microglia-mediated neuroinflammation (63). Furthermore, the exposure of macrophages and microglia to myelin debris *in vitro* leads to an upregulation of A2AR expression in these cells and subsequent CGS21680 treatment inhibits the cellular uptake of myelin debris (35), a well-documented prerequisite for remyelination (64–66). This is corroborated by other studies showing that A2AR stimulation reduces the uptake of fluorescein-labeled *E. coli* bioparticles by LPS-treated microglia (22). Thus, microglial A2AR signaling may be capable of both reducing and exacerbating the severity of EAE due to the complex role of microglial cells in CNS inflammation.

For a long time, investigating the effects of microglia on the progression of EAE has been limited by our ability to distinguish microglia from other myeloid cells. Recently, however, a specific marker of microglia, transmembrane protein 119 (Tmem119), has been identified in both mice and humans, using *in situ* hybridization and qPCR analyses (67). Crucially, FACS studies have shown that Tmem119 distinguishes microglia from infiltrating macrophages in various models of CNS inflammation. Thus, using Tmem119 promotor-driven Cre-recombinase mouse, it may soon be possible to compare the progression of EAE in the presence and absence of microglial A2AR signaling.

EAE IS A USEFUL BUT REDUCTIVE MODEL OF MS

Our understanding of the role of A2AR signaling in the immunopathogenesis of MS is derived almost entirely from MOG-induced EAE studies, which have a number of limitations (5) (**Table 2**).

Most importantly, MS has increasingly been recognized to have a progressive neurodegenerative component that is independent from its autoimmune component and comparable to aspects of Parkinson's disease (4). Accordingly, the greatest limitation of EAE may be its bias toward the immunological component of MS pathophysiology, as illustrated by microarray gene expression profiles, which reveal more changes in immunologically relevant genes in EAE than in MS (68). Indeed, MS has historically been considered a T_H cell-mediated pathogenesis *because* EAE is driven by $CD4^+$ T cells and accurately recapitulates several features of MS. Consequently, the importance of A2ARs in the pathogenesis of MS may be inflated by modeling MS with EAE, where A2AR signaling exacerbates disease *via* its effects on immune cells and immune cell transmigration. However, given the importance of oligodendroglia in regulating remyelination, the recent finding that stimulating surface A1Rs and A2ARs dose dependently causes oligodendroglial death (69) may also implicate A2AR in the neurodegenerative elements of MS.

Indeed, it is possible that neuronal A2ARs might mediate the transition of MS from a disease of neuroinflammation to one of irreversible neurodegeneration. In this regard, A2ARs appear to control the impact of neuroinflammatory mediators on neuronal viability (70, 71) and in different animal models of Alzheimer's disease, A2AR blockade provides neuroprotection at least in part by preventing damage to axon terminals (72, 73). These findings are supported by small-scale clinical studies in which dynamic

TABLE 2 | The limitations of experimental autoimmune encephalomyelitis (EAE).

Feature of EAE	Limitation(s) in recapitulating multiple sclerosis (MS)	Possible solution
Low immunogenic potential of myelin oligodendrocyte glycoprotein (MOG) necessitates administration of strong adjuvants including complete Freud's adjuvant and pertussis toxin	Intense innate immune response to these stimuli in EAE may not reflect pattern recognition in MS	Spontaneous EAE models have been recently established in both the C57BL/6 background and the SJL/J background
MOG-dependent EAE is typically induced in C57BL/6 mice, in which EAE exhibits a chronic, monophasic disease course	Does not reflect the typically relapsing–remitting nature of MS observed clinically	More frequent use of SJL/J strain, which can develop relapsing–remitting EAE
EAE is driven primarily by $CD4^+$ T cells	Underplays roles of $CD8^+$ T cells, which outnumber $CD4^+$ T cells in cortical demyelination lesions in MS, and antigen-experienced B cells, which have been shown to undergo affinity maturation in cervical lymph nodes before migrating to CNS	Corroborate findings with studies using models not driven primarily by $CD4^+$ T cells, e.g., cuprizone feeding and Theiler's virus infection

positron emission tomography imaging of secondary-progressive MS patients, using a radioligand to A2ARs, demonstrate an upregulation of A2ARs in normal-appearing white matter (74). Future studies should investigate the role of A2AR signaling in MS-related neurodegeneration, perhaps using alternative models of MS such as cuprizone feeding (75), which better recapitulates cortical demyelination.

THERAPEUTIC POTENTIAL OF A2A RECEPTORS IN MS

The potential for A2ARs to serve as therapeutic targets in the treatment of MS is frequently alluded to in the literature. For example, administering the A2AR antagonist SCH58261 protected MOG-immunized $A2AR^{+/+}$ tcr-deficient mice from developing EAE both upon the adoptive transfer of WT and upon the transfer of $A2AR^{-/-}$ $CD4^+$ T cells, demonstrating that A2AR antagonist-mediated blockade is protective even in the presence of more pro-inflammatory $A2AR^{-/-}$ $CD4^+$ T cells (23). As discussed earlier, this likely demonstrates the inhibitory effects of A2AR antagonists on non-hematopoietic cells, in which A2AR signaling facilitates the lymphocyte migration into the CNS. However, given that A2AR signaling also downregulates the inflammatory potential of T_H lymphocytes, the potential benefits of inhibiting CNS infiltration cannot be elicited without concomitantly increasing the pro-inflammatory nature of T_H lymphocytes, which risks causing toxic side effects given that adenosine signaling is so widespread in the body and is involved in a range of physiological functions. Indeed, the fine line between the protective and harmful effects of a given A2AR-specific agent has been demonstrated *in vivo*, whereby administering an A2AR agonist on the day of MOG immunization confers protection yet administering the A2AR agonist during the peak of the disease exacerbates EAE (35). Moreover, EAE and MS aside, AR-specific agents have historically struggled to reach the clinic because developing viable AR-specific agents that exhibit tissue selectivity and an appropriate *in vivo* biodistribution is fundamentally challenged by the ubiquity of adenosine signaling in the body (76, 77). In MS, however, the challenge of selectively targeting A2ARs is complicated by the paradoxical effects of these receptors. Thus, targeting A2ARs directly may not have the therapeutic promise that many have hoped for.

Nevertheless, establishing the important role of A2AR-mediated lymphocyte recruitment to the CNS may yield other viable therapeutic opportunities. For example, RT-PCR analysis shows that the expression of CX3CL1 (a chemokine/adhesion molecule) is upregulated in the CPE during EAE (78). CGS21680 also dose dependently increases CXCL1 expression in CPE cell lines. Although it must first be experimentally demonstrated that A2AR-dependent CXCL1 activity increases the infiltration of lymphocytes into the CSF, as indicated by preliminary findings, targeting CXCL1 directly with monoclonal antibodies, rather than *via* A2ARs, may be a viable therapeutic avenue that overcomes the opposing effects of A2AR expression on different cell types. Furthermore, CXCL1 mAb therapies could offer a more precise therapeutic alternative to alemtuzumab, which, while unprecedentedly efficacious, also causes severe immunosuppression that can lead to acquired autoimmune deficiency (79).

As our understanding of the role of T_{reg} A2AR signaling in EAE deepens, this may offer a new avenue for an immunotherapy that is capable of slowing the progression of MS; indeed, there is indirect evidence in support of this. Recently, it was shown that tolerance-inducing gene immunotherapy was able to prevent the onset and progression of MOG-induced EAE (80). By using a liver-targeting gene transfer vector to ectopically express MOG in hepatocytes, functional $FoxP3^+$ T_{reg} cells were induced to expand *in vivo*, and in turn engender tolerogenicity to MOG. This elegant experiment highlights the potential of T_{reg}–related therapies and suggests that the cell types in which A2AR signaling is particularly potent and indeed, demonstrably abrogates CNS inflammation, might offer a therapeutic target more viable than targeting A2AR receptors directly.

Caffeine, a non-selective antagonist of ARs, has been shown to provide protection in MOG-induced EAE. Here, however, it is likely that A2ARs play a minor role in comparison with A1Rs given that caffeine administration is able to reverse EAE pathology in A2AR KO mice and chronic caffeine treatment upregulates A1 receptors but not A2ARs (81). In line with A1R upregulation, an upregulation of TGF-β and a downregulation of IFN-γ mRNA has been observed in Wistar rats induced with EAE (82), consistent with an A1R-mediated shift in T_H1 to T_H2 function. Furthermore, as a readily consumed psychoactive drug, caffeine avoids some of the inherent challenges that AR-specific agents face in reaching the clinic. Epidemiologically, however, the evidence in humans is mixed, with one study showing that caffeine consumption is

not significantly associated with the risk of developing MS (83) whereas a more recent analysis suggests that a high caffeine intake is associated with risk reduction (84). In any case, with respect to possibility of caffeine treatment in humans, A1Rs may be of greater therapeutic importance than A2ARs.

In light of our increasing appreciation of the neurodegenerative component of MS, therapeutic prospects might be informed by insights into other neurodegenerative diseases. It has been shown that excessive A2AR activity is implicated in the development of memory deficits in animal models of Alzheimer's disease (85–87). Consistent with this, both A2AR antagonists and regular consumption of moderate doses of caffeine prevent memory dysfunction arising in a range of conditions, including Parkinson's disease (88), Huntington's disease (89), chronic stress (90), aging (91), early-onset convulsions (92), and diabetic encephalopathy (93). Recently, A2AR inactivation has been found to alleviate early-onset cognitive dysfunction following traumatic brain injury and conditional genetic inactivation of astrocytic A2ARs enhanced long-term memory in the hAPP mouse model of Alzheimer's (94). Thus, even if blocking A2ARs cannot provide a simple solution to managing the progression of MS, it may yet offer some meaningful symptomatic relief and in turn improve patients' quality of life (95).

CONCLUDING REMARKS

In contrast to the anti-inflammatory effects of A2AR signaling in the periphery, which serve to restore tissue homeostasis in response to metabolic stress and cell damage, A2ARs are capable of both facilitating and inhibiting the progression of CNS inflammation. Consequently, A2AR signaling exerts paradoxical effects in the immunopathogenesis of EAE, which in turn undermines the therapeutic potential of these receptors in MS. Even so, unraveling the potent albeit complex effects of A2ARs in EAE, may yet be of instrumental value in revealing novel therapeutic opportunities, which can selectively harness the protective mechanisms induced by A2ARs without targeting these receptors directly.

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Mast Cells and Innate Lymphoid Cells: Underappreciated Players in CNS Autoimmune Demyelinating Disease

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Multiple sclerosis (MS) and its mouse model, experimental autoimmune encephalomyelitis, are autoimmune CNS inflammatory diseases. As a result of a breakdown in the relatively impermeable blood–brain barrier (BBB) in affected individuals, myelin-specific $CD4^+$ and $CD8^+$ T cells gain entry into the immune privileged CNS and initiate myelin, oligodendrocyte, and nerve axon destruction. However, despite the absolute requirement for T cells, there is increasing evidence that innate immune cells also play critical amplifying roles in disease pathogenesis. By modulating the character and magnitude of the myelin-reactive T cell response and regulating BBB integrity, innate cells affect both disease initiation and progression. Two classes of innate cells, mast cells and innate lymphoid cells (ILCs), have been best studied in models of allergic and gastrointestinal inflammatory diseases. Yet, there is emerging evidence that these cell types also exert a profound influence in CNS inflammatory disease. Both cell types are residents within the meninges and can be activated early in disease to express a wide variety of disease-modifying cytokines and chemokines. In this review, we discuss how mast cells and ILCs can have either disease-promoting or -protecting effects on MS and other CNS inflammatory diseases and how sex hormones may influence this outcome. These observations suggest that targeting these cells and their unique mediators can be exploited therapeutically.

Keywords: multiple sclerosis, experimental autoimmune encephalomyelitis, mast cells, innate lymphoid cells, sex-dimorphic autoimmunity, testosterone, meninges, innate immunity

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INTRODUCTION TO MULTIPLE SCLEROSIS (MS) AND EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS (EAE)

MS: A Sex-Dimorphic Autoimmune Disease With a Variable Course

Multiple sclerosis is a CNS demyelinating disease of unknown etiology [reviewed in Ref. (1)]. Although past estimates indicate that this disease affects over 2.5 million people worldwide, more recent studies, reported at the 2017 ECTRIMs meeting, suggest that this is a significant underestimation given that 1 million cases were documented in the US alone[1]. Disease susceptibility is influ-

[1]https://urldefense.proofpoint.com/v2/url?u=https-3A__onlinelibrary.ectrims-2Dcongress.eu_ectrims_2017_ACTRIMS-2DECTRIMS2017_199999_mitchell.t.wallin.the.prevalence.of.multiple.sclerosis.in.the.united.states.a.html-3Ff-3Dmedia-3D3&d=DwIGaQ&c=yHlS04HhBraes5BQ9ueu5zKhE7rtNXt_d012z2PA6ws&r=0eACTVfwt5BhCuBiOTML-EGdMefbkdO179LO68_DpLc&m=_ZQOmVYBDgN9vUsahKvcL5WmWp_exI0RGR0YQAI9oMk&s=wAJJCkTxOI3d0lmbRnsSeIWtATJfdHDBauU35HB2-Mg&e=

enced by a combination of environmental and genetic factors that trigger a chronic autoimmune disorder in which myelin-specific T cells gain access to the CNS through the normally restrictive vasculature of the blood–brain barrier (BBB). Here these cells orchestrate inflammatory damage to the myelin-producing oligodendrocytes, the nerve-insulating myelin sheath and the nerve axons. The resulting loss of saltatory nerve conduction leads to variable neurological dysfunction such as muscle weakness and spasm, loss of motor function and cognitive deficits. Multiple forms of MS exist that are categorized on the basis of variable disease progression, the most common form being relapsing-remitting (RR) MS, accounting for ~85% of cases. In RR MS, transient episodes of clinical symptoms are interspersed with periods of complete or partial remittance, although in many cases RR MS transitions to secondary progressive MS that continues to worsen or reaches a plateau.

Like many autoimmune diseases, MS exhibits a female bias (2–4). The incidence of MS is 3 to 4 times higher in women than in men and women exhibit clinical symptoms at an earlier age and more often experience a RR course. In contrast, men are more prone to develop primary progressive disease in later life. The molecular mechanisms that dictate sex-dimorphism are still largely undefined, yet it is clear that interactions between X chromosome dosage, microbiota, environment, and sex hormones all contribute (3, 5). Both male- and female-dominant hormones are implicated in protection. MS symptoms often improve during late pregnancy and correspond to the high levels of estriol, a hormone proposed to dampen the immune response to the "allo" fetus by generating tolerogenic dendritic cells (DCs) (6). The delayed onset of MS and a more severe disease course in men correlates with the physiologic age-related decline in testosterone, a steroid hormone primarily secreted by the testes (7, 8). Testosterone treatment of male patients can improve MS (9, 10). For example, daily testosterone therapy for 12 months reversed gray matter atrophy and enhanced cognitive performance in a cohort of 10 men with RR MS (10).

EAE: A Rodent Model of MS

Much of what we know about the mechanisms that mediate MS pathogenesis was originally discovered in studies of the rodent model of disease, EAE [reviewed in Ref. (11, 12)]. There are only a few spontaneous models of disease, thus EAE is most often induced by active immunization with myelin proteins or peptides derived from the myelin sheath. EAE can also be elicited by the transfer of encephalitogenic myelin-specific T cells to a naive recipient. The most common models of disease utilize $MOG_{35–55}$-immunized C57BL/6 mice or $PLP_{139–151}$-immunized SJL mice. These mice present with ascending paralysis, and spinal cord involvement is thought to predominate. However, there is significant inflammatory infiltration in the brain with time. In fact immune cell infiltration into the hindbrain precedes the appearance of these cells in the spinal cord in SJL mice. Studies in C57BL/6 mice have been particularly informative because of the many gene deletions placed on this background. Of particular value are the 2D2 mice, which contain a transgene encoding a $MOG_{35–55}$-specific TCR (13). Not only do 2D2 mice facilitate the study of $MOG_{35–55}$-specific responses in EAE but they also provide a useful model to study the spontaneous optic neuritis that occurs in human disease.

SJL mice arguably provide a better model of human disease because these mice develop a relapsing–remitting course that recapitulates the most common form of MS. In addition, similar to humans, there is a marked sex-dimorphism in disease (14). Female SJL mice are more susceptible to EAE, while males, particularly young males, are relatively resistant. There have been considerable efforts to identify the variety of factors that contribute to these sex-determined differences in susceptibility. Studies by Voskuhl and colleagues used the XX and XY^- four core genotype mice to demonstrate that X chromosome dosage is a critical susceptibility factor (15). XY^- mice lack the *Sry* sex-determining region on the Y chromosome and like XX mice are gonadal females, eliminating the potentially confounding influences of hormones. Adoptive transfer of lymph node cells from $PLP_{131–159}$-immunized XX mice to naïve recipients induced significantly more severe disease that XY^- cell transfers implying that XX cells have greater encephalitogenic potential. It is evident that protection in males is not associated with a lack of a myelin-specific T cell response, but rather one that is qualitatively different: whereas females generate a robust encephalitogenic Th1/Th17 response, males produce a Th2 response that is non-pathogenic in this setting (16–19). Differences in basal BBB integrity are also implicated in female susceptibility. SJL females exhibit higher cerebellar expression of the sphingosine-1-phosphate receptor 2 (S1PR2) and signaling through this receptor destabilizes vascular adherens junctions resulting in increased BBB permeability (20).

As in humans, hormones influence EAE susceptibility. Pregnancy is associated with reduced disease symptoms and a link between testosterone and protection has been well established (3, 4). Male SJL mice are more susceptible to disease as they age corresponding with decreasing testosterone levels (21). Testosterone treatment of SJL females attenuates EAE by shifting the pathogenic IFNγ-dominated anti-myelin response to one characterized by the production of IL-4 and IL-10. Expression of other pro-inflammatory cytokines including TNF and IL-1β is suppressed as well (16, 18, 22, 23). Conversely, castration or treatment of male mice with flutamide, an androgen receptor (AR) antagonist, results in increased disease severity (16, 24). Male SJL recipients develop EAE after adoptive transfer of primed T cells from female donors, indicating that testosterone exerts a protective influence during T cell priming (16). However, the precise mechanisms that mediate this testosterone effect have not been completely defined.

THE MENINGES ARE IMMUNE GATEWAYS TO CNS INFLAMMATION

The CNS parenchyma is the main target of immune destruction in MS. However, the meninges, highly vascularized tissues that surround the brain and spinal cord and enclose the cerebrospinal fluid (CSF), serve as critical gateways to local inflammation at these sites [reviewed in Ref. (25)]. The meninges are tripartite tissues that are comprised of the outermost dura mater, which lies directly under the skull or vertebral column; the middle

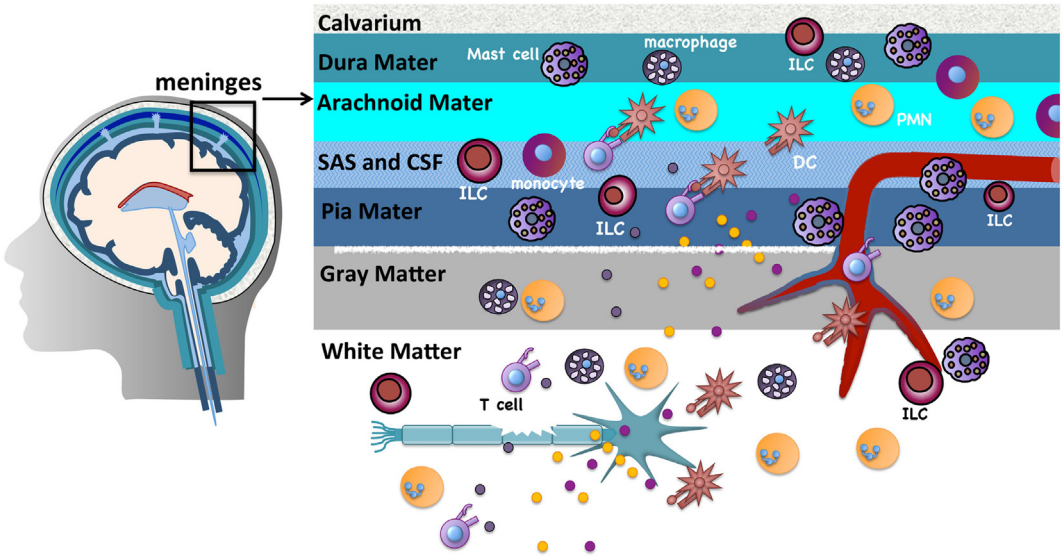

FIGURE 1 | The meninges are sites of active immunity. The meninges are tripartite structures that surround the brain and spinal cord. Multiple innate immune cells reside here. T cells normally transit through the meninges during immunosurveillance and can encounter antigens presented on resident dendritic cells (DCs) or macrophages. Of note, compared to many tissues surveyed, mast cells and innate lymphoid cells are most prevalent in the meninges. These sites serve as first-line protection against infections that threaten the CNS, but are also gateways that can promote chronic CNS inflammation. The inflammatory milieu that is established in the meninges in experimental autoimmune encephalomyelitis/multiple sclerosis is proposed to allow mediator and immune cell infiltration that directly or indirectly damages myelin, olgodendrocytes, and nerves.

arachnoid mater, named for its spider web-like appearance; and the innermost pia mater that often directly adheres to the CNS parenchyma (**Figure 1**). The arachnoid mater and pia mater are collectively referred to as the leptomeninges. In the human brain, the pia mater follows the extensive involutions of the sulci and gyri thus comprising the largest surface area of the three meningeal layers. CSF drains through the subarachnoid space, an anatomical gap between the leptomeninges. Once thought to serve merely as physical protection for the brain and spinal cord, there is increasing evidence that analogous to other "barrier" sites that interface with the external environment such as the skin, airways, and gastrointestinal tract, the meninges function to provide first-line protection against infections that threaten the CNS. Innate immune cells including DCs, macrophages, mast cells, and innate lymphoid cells (ILCs) are normal residents and circulating cells such as neutrophils and T cells transit through the meninges in the course of normal immunosurveillance (26–28). Importantly, lymphatic vessels were recently discovered within the meninges and likely provide a conduit for CNS fluid, immune cells, and macromolecules to access the meninges and the draining deep cervical lymph nodes (29, 30).

Prior to the onset of clinical symptoms in EAE and several days before inflammatory cells are detected in the CNS, there is an influx of peripherally derived immune cells within the meninges (28). Myelin antigens that drain from the CNS to the meninges *via* the CSF are presented by infiltrating or resident APCs to circulating myelin-specific T cells (27, 31–34). The ensuing T cell reactivation results in a local but sustained meningeal inflammatory response that is characterized by cytokine and chemokine production, the influx of additional T cells, neutrophils, and monocytes, and subsequent loss of BBB integrity. Such meningeal inflammation is also observed in MS patients. In patients with progressive MS, T cell infiltrates are found more densely distributed in the meninges than in the CNS parenchyma (35). Ectopic lymphoid follicles, which sequester antigen and facilitate B and T cell activation, have been observed in the meninges of MS patients proximal to cortical demyelinating lesions and their frequency correlates with disease severity (36, 37).

MAST CELLS AND ILCs IN THE MENINGES: INITIATORS OF CHRONIC CNS INFLAMMATORY RESPONSES IN EAE

Immunologists have been aware of the ability of some types of innate immune cells to direct the outcome of B- and T-cell-mediated responses for many years. Cytokines and co-stimulatory molecules expressed by DCs and macrophages, for instance, have established roles in determining T helper cell differentiation fates and B cell isotype switching. Yet, the influence of other classes of innate cells on these processes is not as well defined. Only recently has the potentially potent influence of neutrophils, basophils, and eosinophils been considered in T- and B-cell-mediated diseases, including autoimmune disease. Our laboratory has most recently focused on two additional innate immune cell types that appear to exert profound effects on T cell function in EAE: mast cells and the non-cytotoxic class of ILCs.

Our interest in mast cells in MS/EAE came from several diverse lines of evidence: (1) Mast cells, well known for their

ability to regulate vascular permeability, are juxtaposed with BBB vasculature and often are closely associated with nerves (38). They are also found in the meninges and reside within the CNS where they are most abundant in the thalamus and hippocampus (25, 39, 40). (2) These cells express many effector molecules that have been implicated in disease, including molecules that can directly provoke demyelination (41, 42). (3) Mast cells are found in the demyelinating lesions of MS patients as are transcripts that encode mast cell-associated molecules, such as tryptase, histamine, and FcεR1 (43). (4) Tryptase and histamine are elevated in the CSF of some MS patients, suggesting mast cell activation occurs in disease (44, 45). (5) Drugs that block mast cell degranulation or deplete mast cell granules (proxicromil, cyproheptadine, hydroxyzine) reduce EAE severity (41, 46). Over 17 years ago, we reported that mast cells exacerbated $MOG_{35–55}$-induced disease in female (WB X C57BL/6)F1 mice (47). This finding was subsequently verified in female $PLP_{139–151}$-immunized SJL mice (48). Since that time much of our work has focused on understanding how and where mast cells function to amplify disease severity (28, 49–51). Natural killer (NK) cells, a subset of "cytotoxic" ILCs, have been studied for many years in the context of MS and EAE and have been assigned both pathogenic and protective roles [reviewed in Ref. (52, 53)]. Only in the last 3 years have the non-cytotoxic class of ILCs been implicated in EAE (54, 55).

Like NK cells, there is evidence that mast cells and ILCs can exert either pathologic or protective influence on disease and there is still much to be learned about what determines the nature of their actions in particular settings. Below, we provide a brief overview of the multitude of actions of these cells and discuss what is known about their roles in CNS autoimmune demyelinating disease.

ILC OVERVIEW: POTENT MODIFIERS OF IMMUNE RESPONSES

Innate lymphoid cells comprise a relatively heterogenous group of innate immune cells that include NK cells, which enter the circulation and migrate through tissues, and the non-cytotoxic ILC subsets, Group 1(ILC1), Group 2, (ILC2), and Group 3 (ILC3), most of which remain in tissues and exert their effects locally [reviewed in Ref. (56, 57)]. All ILCs express CD45 and IL-7Rα and share a common lymphoid cell precursor with T and B cells but are lineage negative (lin^-), lacking antigen receptors and other cell surface markers that define T cells, B cells, and myeloid cells.

Natural killer cells were first described in 1975 as important players in protection against viruses and tumors, and have been referred to as the innate counterpart to $CD8^+$ T cells [reviewed in Ref. (56)]. They are defined in part by the expression of the cell surface marker NK1.1, NKG2D, the transcription factors Eomes and T bet, and IFNγ. A $CD56^{bright}$ population of NK cells has been identified only in humans that appear to limits inflammation (52). Despite sharing a common lymphoid progenitor with non-cytotoxic ILCs, NK cells differentiate through a distinct developmental pathway.

With the exception of lymphoid tissue inducer cells (LTis), members of Group 3 ILCs, the non-cytotoxic subsets were not discovered until the early 2000s (56). Since that time non-cytotoxic ILCs have been implicated in a multitude of protective immune responses, chronic inflammation, fat metabolism, and tissue homeostasis. Although relatively rare in tissues at steady state, ILCs respond quickly and vigorously to a wide range of microbial and environmental activating signals by local proliferation and production of effector cytokines in amounts comparable to Th cells (57). MHC Class II^+ ILC2s and ILC3s have antigen presentation activity and thus can also directly interact with $CD4^+$ T cells (58, 59). ILCs are most abundant in mucosal tissues, common regions of pathogen invasion or colonization, but they are also present in non-mucosal sites such as secondary lymphoid organs, the meninges, and the CNS. Although generally considered tissue resident cells, Huang et al. recently described a unique circulating "inflammatory" ILC2 population induced by IL-25 that shows S1P-dependent trafficking to tissues (60).

$CD45^+$ Lin^- IL-$7R\alpha^+$ ILC1s, ILC2s, and ILC3s are analogous to Th1, Th2, and Th17 helper cell subsets, respectively, based on striking developmental and functional parallels and similar to T helper cells, ILCs can also exhibit considerable phenotypic plasticity (57). ILC1s are characterized by the expression of the Th1-determining transcription factor T-bet and produce interferon (IFN)-γ a hallmark Th1 cytokine. ILC2s are $ROR\alpha^+$ and $GATA3^{high}$, both Th2 lineage-determining transcription factors, and express receptors for prostaglandin D2 (CRTh2), and thymic stromal lymphopoietin (TSLPR). They also express ST2, the heterodimeric IL-33 receptor composed of IL1RAcP (IL-1 receptor accessory protein) and ST2 (also known as IL-1 receptor-related protein or IL-1RL1). Upon activation these cells produce the Th2 cytokines IL-4, IL-5, IL-9, and IL-13. The migratory subset of "inflammatory" ILC2s exhibit high expression of KLRG1 and the IL-25 receptor (60). ILC3s are $ROR\gamma t^+$ and express the hallmark Th17 cytokines IL-17, GM-CSF, and IL-22. Functionally distinct subsets of ILC3s are distinguished by the expression of c-kit and membrane-bound lymphotoxin $\alpha1\beta2$ ($LT\alpha1\beta2$), which defines LTis, as well as CCR6, CD4, and NKp46. $Tbet^+$ ILC3 subpopulations have also been described (55). There are differences between human and mouse ILCs, particularly ILC3s. In mice, two subsets are distinguished by CCR6 expression. $CCR6^+$ ILC3s include $CD4^+$ and $CD4^-$ LTi cells and $CCR6^-$ subsets include the $NKp46^+$ and $NKp46^-$ groups. In humans, all ILC3s appear to be $CCR6^+$ and $c\text{-}kit^+$ but show variable expression of NKp44, another natural cytotoxicity receptor (56).

MAST CELL OVERVIEW: MASTERS OF IMMUNE REGULATION

Mast cells are still best known as central players in allergic inflammation, yet these cells are truly multifunctional [reviewed in Ref. (61)]. Mast cells are present at some frequency in most tissues and, depending on their location, demonstrate heterogeneity in the mediators they produce and in cell surface receptors expressed. Thus their response phenotype can vary in a tissue-specific way. Like ILCs, mast cells are most prevalent in tissues that interface with the external environment, the so-called immune border

sites, such as the airways, gastrointestinal and genitourinary tract, and skin where they also contribute to first-line protection against pathogens. Perhaps most densely distributed in the meninges, mast cells, together with other resident innate immune cells, are presumed to have a role in limiting infections that threaten the CNS parenchyma [reviewed in Ref. (25)]. However, mast cells can also contribute to the initiation of chronic inflammation that affects the brain and spinal cord.

A hallmark of mast cells is their ability to store pro-inflammatory mediators such as histamine, prostaglandins, leukotrienes, and certain cytokines in cytoplasmic granules. Within minutes of activation through the high affinity IgE-receptor (FcεR1), mast cell degranulation and release of these effector molecules occurs. Newly synthesized mediators that include cytokines and chemokines are also released in a late phase response. The type of response elicited depends on the site of mast cell degranulation and can range from systemic anaphalaxis to local urticaria, angioedema, and allergic rhinitis. However, mast cells can be activated in a variety of other ways and their contributions are not limited to IgE-dependent immediate type hypersensitive responses. Mast cells express many pattern recognition receptors that interact with conserved microbial molecules (pathogen-associated molecular patterns) or danger-signal associated molecules as well as receptors for cytokines, neuropeptides, complement and hormones. The local millieu of cytokines and chemokines within diverse tissue sites can affect many aspects of innate and adaptive immune cell differentiation, effector function, and trafficking. Mast cells also express MHC Class II, Th polarizing cytokines and co-stimulatory molecules providing the ability to directly interact with T and B cells and regulate their activation response (62–65).

HOW TO STUDY MAST CELLS: MAST CELL LINES AND MAST CELL-DEFICIENT MICE

Mature mast cells do not circulate in the blood and are only diffusely distributed in tissues making the study of these cells notoriously difficult. Many experiments have relied on the use of transformed mast cells or mast cell lines derived from bone marrow precursors after culture in IL-3 and SCF, from precursors in the peripheral blood or mature human foreskin-derived mast cells. Mast cells isolated directly from the peritoneal cavity have also been utilized. In 1978 a mast cell-deficient mouse (WB X C57BL/6)F1-*W/Wv* (WBB6 $Kit^{W/Wv}$) was first described. This phenotype is the result of two distinct spontaneous mutations located within the "white spotting locus," termed *W* and *Wv*, in *Kit*, the gene that encodes the stem cell factor receptor c-kit (66). These mutations do not completely ablate c-kit signaling but cause an ~80–90% reduction of activity. Unlike most hematopoietic cells that only require c-kit signals during early development, mast cells have a strict dependence on vigorous and sustained c-kit signaling for their growth and long-term survival. Differences in a phenotype between wild type and $Kit^{W/Wv}$ mice indicate a possible role for mast cells and thus this mouse model has provided an invaluable tool for defining mast cell contributions to protective and pathologic immunity [reviewed in Ref. (67)]. However, these mice also present considerable obstacles: $Kit^{W/Wv}$ mice are sterile requiring crosses between *W*/+ x *W*^{v}/+ heterozygotes to generate $Kit^{W/Wv}$ mast cell-deficient progeny, and such matings result in only 10–20% of mice with the $Kit^{W/Wv}$ genotype. Numerous other c-kit-related defects including macrocytic anemia, loss of melanogenesis, neutropenia, and altered gut mobility exist in these mice. Thus to confirm mast cell involvement, $Kit^{W/Wv}$ mice must acquire the wild-type phenotype after transfer of bone marrow-derived mast cells (BMMCs), a procedure that selectively reconstitutes mast cell populations but does not correct other c-kit dependent defects.

Other mast cell-deficient mice have since been described that offer some significant advantages over $Kit^{W/Wv}$ mice, although the perfect mast cell-deficient mouse model still does not exist. $Kit^{W-sh/W-sh}$ mice have a mutation upstream from the *Kit* promoter that interferes with c-kit expression (68, 69). These mice are fertile and on a pure C57BL/6 background, but have increased numbers of splenic mast cell precursors, basophils, and neutrophils, lack melanocytes, and exhibit a time-dependent loss of mast cells (70). $Cpa3^{Cre/+}$ mice, described in 2011, contain a transgene encoding Cre-recombinase under the control of the carboxypeptidase 3 (*Cpa3*) promoter (71). High expression of Cre-recombinase in carboxypeptidase 3-expressing mast cell precursors is toxic, causing genomic instability and the ultimate demise of the mast cell lineage at an early stage in development. These so-called "Cre-master" mice also have reduced numbers of basophils, a cell type that shares a common *Cpa3*-expressing precursor with mast cells, but other hematopoietic cells do not appear to be affected. Lilla and colleagues reported that *Cpa-3-Cre;Mcl-1*$^{fl/fl}$ mice exhibit a profound reduction in mast cells in most tissues with the exception of the spleen, but are also anemic, have reduced basophils and are neutrophilic (72). Mast cell protease 5 mice (*Mcpt*-5)-*Cre;R-DTA*$^{fl/fl}$ mice have a loss of most peritoneal and skin mast cells, but largely retain mucosal mast cell populations (73).

MAST CELLS AS AMPLIFIERS OF CNS INFLAMMATION

Despite their limitations, $Kit^{W/Wv}$ mice on the (WB X C57BL/6) F1 and SJL backgrounds have been extremely informative in our studies of mast cell contributions to EAE. Female $Kit^{W/Wv}$ mice of both strains develop significantly less severe EAE than their wild-type counterparts, a phenotype that is reversed by selective mast cell reconstitution (47, 48). Restoration of mast cells to the meninges alone is sufficient for restoring wild-type disease severity, indicating that mast cells residing in these CNS-proximal tissues are a relevant population in regulating disease onset (51). Indeed, meningeal mast cells are activated within a day after active or passive EAE induction and express many pro-inflammatory genes implicated in disease, including *Tnf*, *Il1b Cxcl1*, *Cxcl2*, as well as mast cell-specific genes encoding proteases such as *Cpa3*, *Mcpt2*, and *Tpsab1* (49).

Mast cell-mediated influence on disease severity appears to operate at several levels (**Figure 2**):

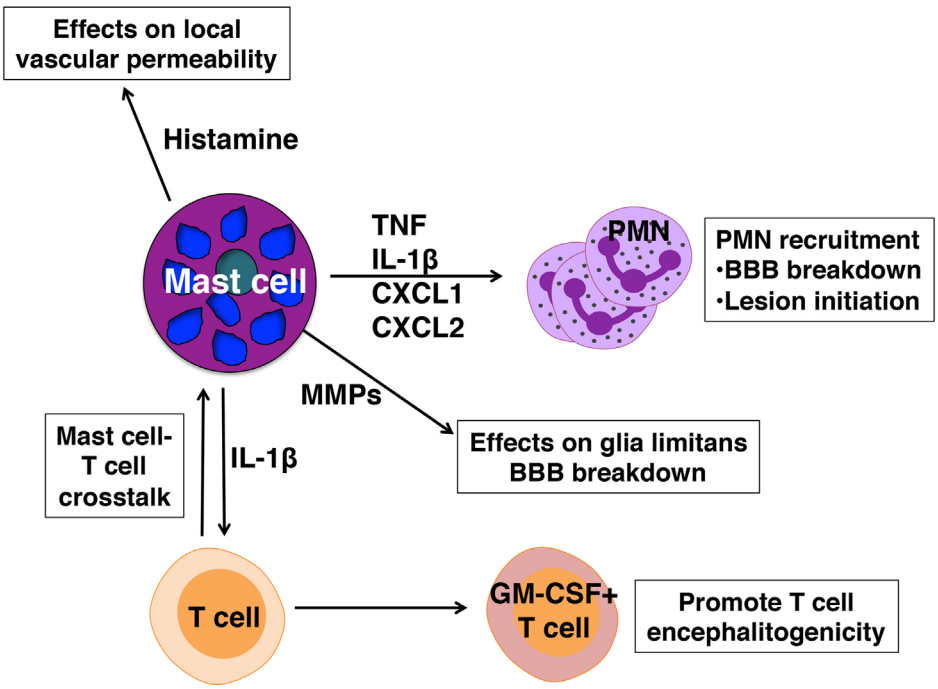

FIGURE 2 | Pathogenic actions of mast cells in experimental autoimmune encephalomyelitis. Mast cells are activated in early disease and express mediators that affect blood–brain barrier (BBB) integrity and license T cells for pathogenicity.

(1) Mast cells located in the meningeal pia mater are situated in close proximity to BBB vasculature and as in peripheral blood vessels, can affect local vascular permeability. In the absence of mast cells frank BBB permeability is not attained (49). As a result, although T cell transit through the meninges is similar in wild-type and *Kit*$^{W/Wv}$ mice, autoreactive Th1 and Th17 cells do not efficiently access the CNS parenchyma in *Kit*$^{W/Wv}$ mice (28, 50).

(2) We have demonstrated that mast cells are essential for the early and relatively robust recruitment of neutrophils to the meninges and CNS, an event dependent on TNF expression by mast cells (28, 49). Neutrophil-related markers, including CXCL1, are increased in the blood at clinical onset of EAE and not only are neutrophils necessary for disruption of BBB integrity (74), they likely have a role in early lesion initiation in the CNS as shown in human studies (75). CXCL5 and neutrophil elastase are elevated in MS patients with active disease and correspond with the presence of acute lesions detected by MRI. Furthermore, plasma levels of CXCL1, neutrophil elastase, CCL2, and CXCL5 in these patients correspond with expanded disability status scale scores, a measure of disease disability.

(3) Mast cell-T cell interactions in the meninges promote T cell encephalitogenicity as well. It has been demonstrated that autoreactive Th17 and Th1 cells primed in peripheral lymphoid organs are not inherently pathogenic, but acquire disease-causing function during their transit to the CNS, a process termed "T cell licensing." Odoardi et al. showed that myelin-reactive T cells traffic from the lymph nodes through the lungs and acquire new gene expression, including genes encoding molecules that assist in transendothelial migration (76). Our work revealed that interactions between resident mast cells and autoreactive T cells in the meninges induce caspase-1-dependent IL-1β production by mast cells, which in turn elicits T cell production of GM-CSF (50). GM-CSF is a cytokine essential for T cell encephalitogenicity that acts to recruit CCR2^{+} inflammatory monocytes into the CNS (77–80). In the absence of meningeal mast cells or IL-1β production by mast cells, T cell GM-CSF production is reduced, as is EAE severity (50).

Despite evidence in both rodent and human studies that lends credence to the idea that mast cells are critical players in EAE and MS, some experiments with mast cell-deficient mice have not supported such a role [reviewed in Ref. (81)]. There are two striking examples: (1) C57BL/6 *Kit*$^{W-sh/W-sh}$ mice develop more severe disease than their wild-type cohorts (82) and (2) Although *Cpa3*$^{Cre/+}$ mice are refractory to induction of IgE-mediated mast cell disease, no differences in EAE disease severity were observed in side-by-side experiments with wild-type, *CPA3*$^{Cre/+}$, and *Kit*$^{W/Wv}$ mice (71). The reasons for these experimental discrepancies are not clear, but likely reflect the fact that by definition, autoimmune diseases like EAE require T and/or B cells. Thus mast cells can only serve an accessory role. Disease induction conditions that elicit early and strong T cell responses in EAE models, conditions that do not recapitulate the normal evolution of MS in humans, can mask the contributions of mast cells. In support of this idea, Piconese et al. showed that altering disease inducing conditions

in $Kit^{W/Wv}$ mice changed the apparent mast cell dependence on severe disease development (82). Furthermore, in experiments comparing disease in $Kit^{W/Wv}$ and $Cpa3^{Cre/+}$ mice early and high morbidity was observed in all groups indicating that a very strong T cell response was elicited during EAE induction (71). The inherent inflammatory state of in $Kit^{W-sh/W-sh}$ mice, reflected in increased steady state numbers of neutrophils, may also mitigate the requirement for mast cell-dependent neutrophil recruitment that affects early disease-promoting events.

Kit$^{W/Wv}$ MICE REVEAL A MAST CELL-ILC2 CONNECTION THAT PROVIDES MALE-SPECIFIC PROTECTION IN EAE

Studies of EAE in SJL *Kit* mutant mice have also provided insights into the molecular mechanisms that underlie sex-dimorphic EAE in SJL mice. Surprisingly, in contrast to SJL $Kit^{W/Wv}$ females, in which the *Kit* mutations protect from severe EAE, male SJL $Kit^{W/Wv}$ mice develop severe clinical symptoms (83). They also generate a predominant Th17 anti-myelin response rather than the Th2-dominated response of wild-type males. Importantly, mast cell reconstitution of $Kit^{W/Wv}$ males does not restore protection. While this finding cannot eliminate a role for mast cells, it does indicate that another c-kit$^+$ cell type is required for protection. Indeed, it was subsequently discovered that $Kit^{W/Wv}$ mice have profound defects in ILC2 development and fail to show the increases of ILC2 numbers in the draining lymph nodes, brain, and spinal cord that are characteristic of immunized wild-type males. Because ILC2s are essential for initiating and maintaining Th2 immunity (84, 85), we hypothesized that diminished ILC2s in male $Kit^{W/Wv}$ mice are responsible for the lack of a strong Th2 response. These findings raised the possibility that Th17 response-prone females also have defective ILC2 responses. To test this ILC2 numbers in the draining lymph nodes and CNS of $PLP_{139-151}$-immunized males and females were compared and, as predicted, found to be significantly lower in females (19). This muted response is not due to an intrinsic defect in female-derived ILC2s: ILC2 development is comparable in females and males as is the ability of ILC2s to respond to exogenous inflammatory cues delivered both *in vitro* and *in vivo*.

DECREASED IL-33 EXPRESSION LIMITS ILC2 ACTIVATION AND PROMOTES SUSCEPTIBILITY IN SJL FEMALE MICE

ILC2s can be activated by a number of mediators including TSLP, IL-25, prostagladin D2, IL-7, and IL-33 (86). Among these IL-33, a multifunctional cytokine belonging to the IL-1 superfamily of cytokines, is considered to be the most potent (87). Produced constitutively by epithelial and endothelial cells, DCs, macrophages, and several CNS resident cells, IL-33 is often localized in the nucleus and acts as an alarmin when passively released by damaged or necrotic cells [reviewed in Ref. (88)]. Yet IL-33 is inducibly expressed in certain inflammatory settings through, for example, autocrine activation of P2 purinergic receptors (88–90). We observed that $PLP_{139-151}$-immunized SJL females exhibit significantly reduced induction of *Il33* in multiple tissues when compared to male mice, providing an explanation for their reduced ILC2 activation response. Collectively our data suggest that, similar to its actions in helminth infections and allergic responses (86), IL-33 signals ST2$^+$ ILC2s to produce IL-13 and other Th2 polarizing cytokines, which in turn promote a Th2-dominated response in males, one that is non-pathogenic in the context of EAE (**Figure 3**). It is likely that other ST2$^+$ cells are targets of IL-33 and act in concert with ILC2s to confer male-specific protection. Mast cells and basophils also produce Th2 cytokines when stimulated with IL-33 (88) and an ST2$^+$ T regulatory cell subset has been defined that demonstrates an IL-33-dependent ability to limit inflammation in a model of inflammatory bowel disease (91). Notably, Matejuk et al. show that decreases in Foxp3$^+$ Tregs correspond with declining testosterone levels in aging C57BL/6 mice with severe EAE, although IL-33 was not measured in these studies (24).

The critical role of IL-33 in EAE resistance was confirmed in experiments in which administration of IL-33 to female mice prior to immunization elicits ILC2 activation and prevents disease (19). Strikingly, even when given at the peak of clinical symptoms, IL-33 prevents relapse by inducing ILC2 activation in the meninges and CNS and converting an established Th17-dominated response to one that is Th2. Antibody blockade of IL-33 abolishes EAE protection in males.

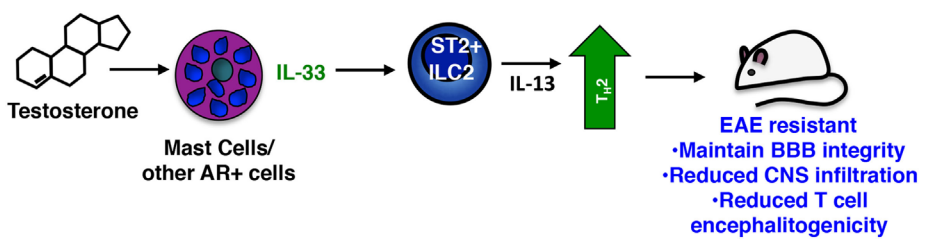

FIGURE 3 | Sex-specific protective actions of mast cells and innate lymphoid cells (ILCs) in male SJL mice. In the sex-dimorphic model of multiple sclerosis in SJL mice, males are protected. Testosterone activates mast cells [and perhaps other androgen receptor (AR)$^+$ cells] inducing IL-33, which in turn activates the Th2-promoting action of ST2 + ILC2s. These cells limit the Th17-dominated response characteristic of susceptible females and drive a non-pathogenic Th2 anti-myelin response.

A CONTEXT-DEPENDENT *PROTECTIVE* FUNCTION OF MAST CELLS IN EAE

It was reported that the mast cell-specific proteases chymase and tryptase generate a mature form of IL-33 with increased ability to activate ILC2s (92). Thus it is intriguing that mast cells are one important source of IL-33 in male SJL mice. *Il33* induction is significantly diminished in the lymph nodes, meninges and CNS of $PLP_{131–159}$-immunized male SJL $Kit^{W/Wv}$ mice, a response largely restored by mast cell reconstitution (19). IL-33 protein is also detected in meningeal mast cells. The mechanism of IL-33 release is still unknown, but similar to cytokines such as TNF, IL-33 may be actively transferred to cytoplasmic granules and released through degranulation (93, 94).

The differential IL-33 response in males and females led to the obvious question: does testosterone influence expression? Serum testosterone levels show an early and transient increase in immunized males and male-derived mast cells express higher levels of the AR *ex vivo*. Direct evidence for testosterone actions on IL-33 induction in mast cells come from studies with male- and female-derived BMMCs (19). Although there is no sex-dependent difference in AR expression in these cultures, testosterone selectively induces *Il33* in male-derived bone marrow mast cells. This male-specific response is also observed in IgE- and Mycobacterium-activated cells, and we speculate that testosterone can affect IL-33 production in at least two ways: (1) Upon immunization, increasing serum levels of testosterone directly drive the IL-33 response by mast cells and other AR^+ IL-33 expressing cells (**Figure 3**); and (2) Long-term exposure to this hormone may alter the chromatin landscape during mast cell development, poising this locus for expression in males and repressing it in females. Of note, females also express a unique subset of cytokines that are not induced in males, including TNF and IL-1β, after immunization indicating a striking context-dependent effect on mast cell responses likely regulated by hormones.

Pronounced sex-dimorphic EAE susceptibility is only observed in certain strains of mice (14), suggesting the testosterone-driven IL-33/ILC2 pathway that functions to protect in SJL males does not operate in all strains. This could be due to the documented variations in serum testosterone levels, some of which are potentially too low to trigger this pathway (95). A side-by side analysis of serum testosterone levels in resistant SJL and susceptible C57BL/6 males revealed significantly lower levels in C57BL/6 mice consistent with this idea (19). It will be interesting to determine whether this pathway functions in EAE resistant strains in which both sexes are protected, such as BALB/c and if so, why it is not sex dependent. Obviously, other factors such as strain specific differences in mast cell numbers and cytokine responses also may play a role in differing susceptibilities (96, 97).

NON-CYTOTOXIC ILCs IN EAE/MS PATHOGENESIS

In contrast to the apparent protective influence of ILC2s in EAE, ILC1s and ILC3s are implicated in promoting severe EAE. Much of the data to support this claim are correlative and reflect the fact that many features of these cells are consistent with a pathogenic role in disease (**Figure 4**). Like ILC2s, ILC1s, and ILC3s are normally present in the meninges and CNS and increase in number after disease induction (54, 55). When activated these cells produce IFNγ, IL-17, GM-CSF, and other cytokines that have been linked to EAE pathogenesis. Myelin-specific $CD4^+$ T cells with a memory phenotype are the major pathogenic T cell population in EAE (98) and ILC3s express CD30L and OX40L, molecules that promote the maintenance of memory T cell function (99). The LTi subset of ILC3s express relatively high levels of IL-17 and IL-22, as well as membrane-bound LTα1β2 and may also be relevant in disease (100, 101). LTis are critical for the normal development of lymph nodes and Peyer's patches, functions dependent on LTα1β2, but also drive ectopic lymphoid follicle formation (eLFs) in non-lymphoid tissues. Although eLF formation in response to persistent bacterial and viral infections are protective, these structures, which range from aggregates of diffuse T and B cells to those that resemble conventional lymphoid organs, are hallmarks of the chronic inflammation associated with several autoimmune diseases including MS [reviewed in Ref. (101)]. It has been hypothesized that these follicles form the framework for the sequestration and presentation of autoantigen to T and B cells as well as for intrathecal (subarachnoid) antibody production, another distinguishing feature of MS (102). Notably ectopic lymphoid follicles are found closely associated with inflamed vasculature within the leptomeninges where ILCs reside. Treatment with an antagonist of LTβ blocks the formation of meningeal eLFs and also significantly suppresses EAE onset as well as severity (103).

An incontrovertible role for ILCs in EAE has been difficult to prove because, as for mast cells, there is no selective ILC2-knockout mouse. Mair and Becher were among the first to examine a possible ILC contribution to EAE using a cell depletion approach (104). ILCs, defined as $Thy1^+$ $Sca1^+$ cells, are increased in the CNS of C57BL/6 mice with EAE. Yet depletion using anti-Thy1 antibodies had no effect on limiting disease severity, suggesting either that ILCs are not important or that Thy1 cell depletion does not target the pathogenic ILC population. Our laboratory observed that adoptive transfer of encephalitogenic Th17 cells to $Rorc^{-/-}$ mice, lacking both ILC3s and the ability to generate Th17 responses, is not sufficient to induce disease indicating that ILC3s are also required (54). In perhaps the most definitive demonstration of a pathogenic role for ILCs in EAE, Kwong et al. showed that adoptive transfer of $MOG_{35–55}$-specific 2D2 transgenic T cells into mice with a hematopoietic cell-specific deletion in *Tbx21*, are not susceptible to severe disease (55). *Tbx21* encodes T-bet, a transcription factor critical for Th1 differentiation and these findings revealed that in addition to T cells, other $Tbet^+$ immune cells are candidates for influencing disease. Using a series of mice with DC- NK- and NKp46-specific deletions in *Tbx21*, the investigators narrowed the critical population to $Tbet^+$ $NKp46^+$ ILC1s and ILC3s that primarily reside within the meninges. In their absence, T cell influx to the CNS and disease development are significantly reduced. Although the precise mechanism of $Tbet^+$ ILCs' action has not been determined, these cells are purported to function by orchestrating the expression of cytokines,

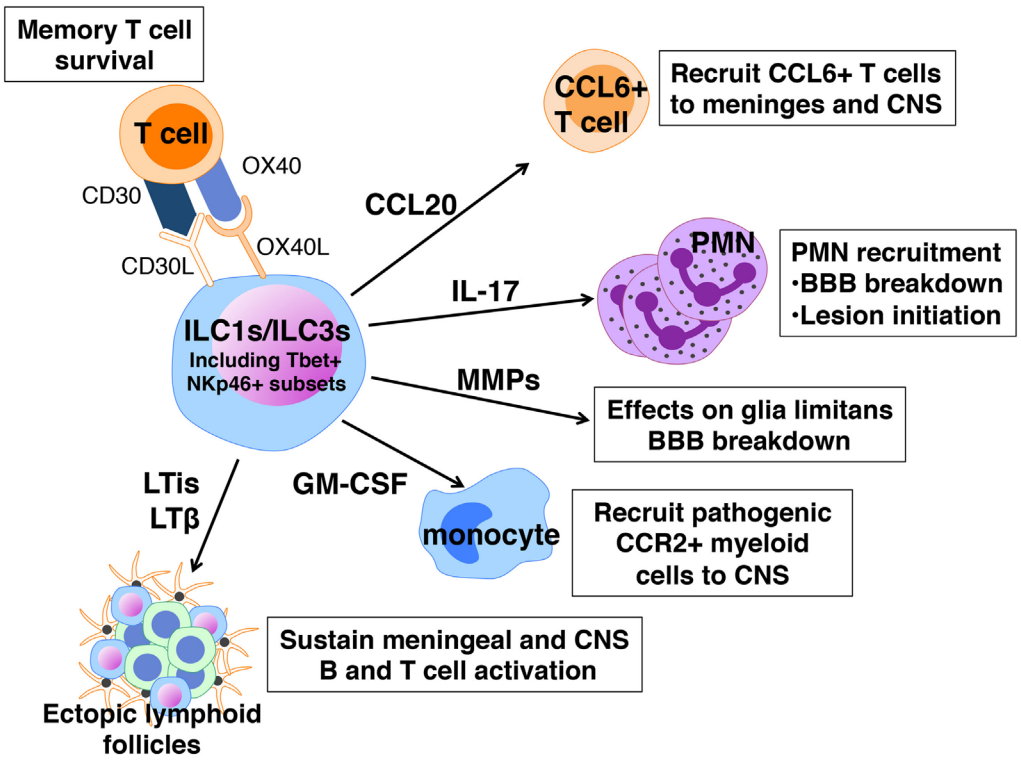

FIGURE 4 | Pathogenic actions of innate lymphoid cells (ILCs) in experimental autoimmune encephalomyelitis. ILC1s and ILC3s express a number of cell surface receptors and mediators that can sustain memory T cells, cause local loss of blood–brain barrier (BBB) integrity, and recruit neutrophils, T cells, and myeloid cells to the meninges and CNS.

chemokines, and matrix metalloproteinases within the meninges, many of which regulate BBB integrity and cell recruitment to the CNS. For example, the expression of CCL20, which is needed for the entry of $CCR6^+$ T cells to the CNS parenchyma and MMPs that break down the glia limitans are much reduced in *Tbx21*$^{f/f}$ $NKp46^{Cre/+}$ mice.

NK CELLS: ANOTHER CELL TYPE WITH DUAL EFFECTS ON MS/EAE OUTCOMES

Natural killer cells have also been implicated in EAE and MS although again, it is unclear whether they exert a beneficial or detrimental role. The answer is probably both give the identification of distinct subpopulations with cytotoxic and "regulatory" functions and cytokine-producing abilities [reviewed in Ref. (52, 53)]. Differences in human and mouse NK cell markers and the lack of selective NK cell-deficient mice limit progress in analyzing these cells in EAE models. $CD56^{dim}$ cells are considered the major cytotoxic NK population and a role in pathogenesis has been proposed due to their ability to activate CNS-infiltrating DCs, assist in Th1 polarization and kill oligodendrocytes, astrocytes and microglia *in vitro*. However, most studies suggest NK cells are beneficial. In mice, cytotoxic NK cells are proposed to "edit" pathogenic lymphocytes and macrophages by direct cytotoxicity or through the production of regulatory cytokines. NK cells produce neurotropic factors such as brain-derived neurotropic factor and neurotropin-3, consistent with a role in neural repair. The most convincing evidence for NK-mediated protection comes from human studies. Immune cell profiling of healthy volunteers and untreated patients with clinically isolated syndrome or RR MS revealed a reduction in the frequency of an NK-like population ($CD3^-$ $CD56^+CD8^{dim}CD4^-$) in MS patients. Importantly daclizamab therapy (directed to IL-2 receptor α) results in an increase in this population. The increase of a $CD56^{bright}$ subset of NK cells in treated patients, corresponds with the inhibition of contrast-enhancing brain lesions seen in MRI.

CONCLUDING REMARKS

Multiple sclerosis is generally considered to be a T cell-mediated disease although the surprising efficacy of anti-CD20 B cell depleting drugs, such as rituximab, ocrelizumab, and ofatumumab indicate B cells also have an important but as yet unclear role in disease pathogenesis (105). Most targeted therapies have been directed at altering T cell function or migration. The realization that neutrophils, mast cells, and ILCs can exert profound influences on T cell polarization, effector function, and immune cell infiltration to the CNS suggests that new therapeutic strategies should be considered that also target these cells and their mediators. These findings provide strong rationale for studies to continue exploring the T cell modulating roles of mast cells, ILCs and neutrophils in EAE and MS.

Eosinophils and basophils are also worth further examination. Eosinophils are potent cytokine-expressing cells that can serve as APCs and it has been proposed that they may help re-educate pathogenic T cells toward an anergic state (106). In parasitic infections, eosinophils are a major effector cell population and patients with MS who are infected with helminths show less severe disease than uninfected cohorts (107). This observation has been replicated in mice in a study showing that administration of helminth derived products limits EAE severity (108). Protection was dependent on IL-5 and IL-33 and the expansion of eosinophils. Basophils, prolific producers of IL-4 and other Th2 cytokines, are also promising targets. Long considered the "circulating counterpart" of tissue resident mast cells, these (c-kit$^-$ FcεR1$^+$) cells also have preformed granules that contain a variety of allergic mediators that are released upon IgE cross-linking. Yet the maturation, life span, and unique gene expression profile indicate these cells are distinct (109). Basophils are clearly linked to pathogenesis in a Lyn kinase-deficient mouse model of systemic lupus erythamatosis (110). In this model, development of disease is dependent on the production of IgE autoantibodies. Depletion of basophils limits IL-4 production, reduces IgE, reverses the Th2 bias in these animals and improves the characteristic glomerulonephritis, caused by antibody complex deposition. The strong Th2 cytokine response of basophils would predict that basophils might be protective in MS and EAE and a recent study suggests this may be the case. Anti-FcεR1 treatment depleted basophils and worsened disease in B6 mice and conferred susceptibility to the normally resistant Th2 prone BALB/c mice (111).

There are several lessons to be learned. First, there is now much independent evidence that shifting the pathogenic Th response in MS and EAE to a Th2-dominated one is a viable therapeutic approach and there are a number of drugs currently used in treatment including dimethyl fumerate and glatiramer acetate that have this effect. However, there may be more powerful ways to achieve this outcome by harnessing innate cells; mast cells (in some settings), ILC2s, eosinophils, and basophils, whose physiologic functions in infection are linked to promoting strong Th2 responses. We also need to rethink current therapies that were developed to specifically target T cells but may exert more far-reaching and perhaps unwanted effects. An important example is Fingolimod (FTY720, Gilenya), an S1P receptor antagonist that induces receptor internalization. Because T cells require S1PR signaling to migrate from secondary lymphoid organs to tissues, they are sequestered in the lymph nodes. This drug was also shown to directly affect the S1PR2-expressing BBB endothelium. Blockade of S1P signals increases BBB integrity and limits EAE severity (20). What has not been appreciated is the potential effects of this blockade on other innate immune cells. The circulating KLRG^{hi} "inflammatory" subset of ILC2s are activated by IL-25 and show S1P-dependent migration to tissues (60). Fingolimod treatment abrogated the protective ILC2 mediated responses in a helminth infection model. Thus, although this drug is relatively efficacious in reducing time between MS relapses, inhibition of a Th17-modulating ILC2 response, if shown to be functional in humans, may be undesirable in some patients. Fingolimod also reduces circulating CD56^{bright} NK cells, considered to be protective and such effects may be undesirable (52). In macrophages S1P signaling shifts pro-inflammatory M1s to the anti-inflammatory M2 phenotype, an effect that may be desired in MS (112). Mast cells express S1PRs and are a potent source of S1P and their biology is intimately tied to this signaling pathway [reviewed in Ref. (113, 114)]. It is difficult to predict the effects of Fingolimod treatment on mast cells in the context of MS. Chronic S1P exposure induces the differentiation of a mast cell that is hyperresponsive to IgE-receptor signaling and S1PR2 deficient mast cells show reduced degranulation responses after ATP or phorbol ester/ionomycin activation.

Another class of drugs, receptor tyrosine kinase inhibitors such as imatinib (Gleevac), have been proposed for use in MS treatment (115). Originally used to treat certain cancers, imatinib inhibits the TCR/Abl tyrosine kinase signaling pathway and limits T cell cytokine responses. BCR-Abl-kinase and c-kit signaling are also inhibited by imatinib, suggesting it may be useful to target mast cells in MS. Imatinib blocks mast cell proliferation *in vitro* and attenuates the onset and severity of EAE in both a rat and mouse model of disease (116, 117).

There are several challenges as we go forward. First and perhaps most importantly, we must verify that innate immune cells have similar immunomodulatory effects in human disease. Although the data implicating mast cells and ILCs in EAE is intriguing, there is still is a paucity of information regarding a role for these cells in MS. As discussed earlier mast cells are normally present in the human meninges and brain and are associated with demyelinating lesions. Meningeal tissues from a small cohort of acute MS patients provide evidence of meningeal mast cell-T cell co-localization associated with areas of subpial cortical demyelination (50). Mast cells were also observed in white matter parenchymal lesions of differing demyelinating stages (early active, inactive, and remyelinated), often in close proximity to infiltrating T cells. Even less is known about non-cytotoxic ILCs in MS and this information comes from drug studies in which CD25 blockade reduced numbers of the circulating LTi subset of ILC3s and inhibited meningeal inflammation (118).

In view of the dramatic sex and strain differences in murine responses, we also must consider the possibility that the diverse genetic and environmental backgrounds of humans will lead to variable innate immune cell responses. Thus in some cases we may want to inhibit innate immune cell activation (e.g., in settings where mast cells are pathogenic), while in others, we may want to enhance their activation (e.g., in cases where mast cell production of anti-inflammatory mediators like IL-33 occurs).

Finally, the ultimate goal of all research in autoimmune disease including MS is to develop therapeutic strategies that stop disease progression and confer a lasting cure as well as eliminate the common generalized immunosuppression often associated with current treatments. Our data demonstrating IL-33 can prevent relapses even when administered to mice at the peak of established disease are promising, particularly in view of its actions on multiple cell types. Future studies need to determine whether

direct administration of IL-33 to MS patients or use of strategies to enhance endogenous production may be effective in reversing MS symptoms, without dangerous side effects. IL-33 was shown to have direct effects on oligodendrocyte gene expression and induce p38 MAPK phosphorylation these cells, an event linked to myelination (119) indicating that treatment with this cytokine or therapies directed downstream of IL-33 may fulfill a dual role of blocking damaging inflammation as well as promoting myelin repair.

AUTHOR CONTRIBUTIONS

MB and RW wrote and edited the manuscript.

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MINI REVIEW
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Evaluation of Microglial Activation in Multiple Sclerosis Patients Using Positron Emission Tomography

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Understanding the mechanisms underlying progression in multiple sclerosis (MS) is one of the key elements contributing to the identification of appropriate therapeutic targets for this under-managed condition. In addition to plaque-related focal inflammatory pathology typical for relapsing remitting MS there are, in progressive MS, widespread diffuse alterations in brain areas outside the focal lesions. This diffuse pathology is tightly related to microglial activation and is co-localized with signs of neurodegeneration. Microglia are brain-resident cells of the innate immune system and overactivation of microglia is associated with several neurodegenerative diseases. Understanding the role of microglial activation in relation to developing neurodegeneration and disease progression may provide a key to developing therapies to target progressive MS. 18-kDa translocator protein (TSPO) is a mitochondrial molecule upregulated in microglia upon their activation. Positron emission tomography (PET) imaging using TSPO-binding radioligands provides a method to assess microglial activation in patients *in vivo*. In this *mini-review*, we summarize the current status of TSPO imaging in the field of MS. In addition, the review discusses new insights into the potential use of this method in treatment trials and in clinical assessment of progressive MS.

Keywords: microglia, positron emission tomography, imaging, 18-kDa translocator protein, multiple sclerosis

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INTRODUCTION

Multiple sclerosis (MS) is a chronic autoimmune disease of the central nervous system (CNS) which leads to demyelination and neurodegeneration. In 85% of cases, MS starts as a relapsing remitting disease following an attack against the CNS by the adaptive immune system. This leads to formation of MRI-detectable, gadolinium-enhancing focal inflammatory lesions. Depending on the anatomical location of the lesions, neurological symptoms, i.e., MS relapses, will follow. Inflammation within the CNS contributes to demyelination and neuronal damage (1). Within 10–15 years after the diagnosis, more than 60% of RRMS patients procede to develop secondary progressive MS (SPMS) in which relapses give way to relentless disease progression and accumulation of disability (2). This progression is associated with activation of the local innate immune system within the CNS and, gradually, white blood cell trafficking from the periphery into the CNS is reduced (3). Both resident microglia and blood-derived macrophages contribute to neuronal damage *via* release of pro-inflammatory cytokines and reactive oxygen species (4). These lead to oxidative injury of mitochondria and to oligodendrocyte damage and degeneration of neurons (5, 6). The resulting energy failure and membrane channel dysfunction may be key processes in progressive disease. Interfering with these mechanisms,

for example by reducing the harmful pro-inflammatory microglia functions, may provide neuroprotection and prevent disability progression by myelin repair and restoration of axonal activity and conduction.

Neuropathological studies have demonstrated that MS lesions in progressive disease rarely have features of acute inflammation. Instead, brain samples from patients with progressive disease have chronic active (smoldering or expanding) lesions with microglial activation at the edge of an otherwise burned out plaque (7). Alternatively, the chronic lesions are inactive, with no microglial activation at the plaque edge (7). In addition, widespread microglial activation is seen in areas surrounding the focal lesions, in the so called normal-appearing white matter (NAWM) (8). Microglial activation is associated with signs of neuronal damage and tissue atrophy and hence it is assumed that microglial cells contribute to the CNS damage of progressive MS (9). In this narrative *mini-review*, we give a comprehensive overview of the present state of the use of positron emission tomography (PET) using 18-kDa translocator protein (TSPO)-binding radioligands for imaging of microglial activation in MS. We have used PubMed for literature searches using the following search terms: TSPO imaging, neuroinflammation, PET, and MS. We discuss the promise and potential of TSPO imaging in *in vivo* visualization of microglial activation in association with various aspects of MS, address significant gaps in the field and highlight future directions for further investigation.

WHY NEW IMAGING METHODS ARE NEEDED FOR THE STUDY OF MS?

Given the current limited understanding of the neuropathological process of progressive MS, it is not surprising that the disease modifying treatments used successfully to treat RRMS, which mostly function on the peripheral adaptive immune system, are not effective for progressive MS. Attempts to find treatments for progressive MS have proven challenging with, frequently, disappointing results (10). However, recently, ocrelizumab, a humanized monoclonal antibody selectively depleting CD20-expressing B-cells, was the first disease modifying treatment to show efficacy in slowing down disease progression in primary progressive MS (11). A breakthrough is still awaited for effective treatment of SPMS. Imaging methods or biomarkers for progressive MS, which would assist in treatment development, are not well established and the diagnosis is usually retrospective, based on the history of gradual neurological worsening with or without occasional relapses (12). Conventional MRI is sensitive in demonstrating the gadolinium enhancing active focal inflammatory lesions, and MRI is essential for MS diagnostics, clinical follow-up and treatment trials of RRMS. MRI studies in progressive MS, on the other hand, often demonstrate limited blood–brain-barrier (BBB) permeability. This is in accordance with the ongoing compartmentalized inflammation within the CNS which has been well demonstrated in progressive disease using neuropathological studies (13). Other MRI characteristics of progressive MS include increasing number and volume of T1-hypointense lesions, brain volume loss, changes in magnetic transfer imaging, and diffusion tensor imaging (14). Conventional MRI is not sensitive enough to visualize the diffuse pathology associated with progressive MS. Hence, more sensitive methods for monitoring progressive MS are urgently needed. PET imaging using radioligands binding to the TSPO molecule on activated microglial cells provides a method to specifically quantify microglial activation both in the context of the chronic lesions and within the NAWM. PET imaging will enable longitudinal *in vivo* follow-up of the pathobiology relevant to progressive MS, and it thus holds promise as a new outcome measure for treatment studies of this under-treated condition.

DESCRIPTION OF THE PET METHODOLOGY

Positron emission tomography imaging uses short-lived radioactive isotopes bound to ligands that interact with their specific targets within the CNS (15). The radioactive isotopes emit positrons, that are detected using a sophisticated gamma-counter placed within a PET camera, and the amount of the bound ligand within the CNS can thus be quantitated. Radioligands used for PET imaging are produced by radiolabeling specific precursor molecules (the receptor ligands) with short-lived positron emitting isotopes, such as ^{18}F and ^{11}C using a cyclotron. Due to the short half-lives of the tracers, i.e., 20 min for a ^{11}C-tracer or 110 min for a ^{18}F-tracer, a short cyclotron-to-camera-time is required, and the radioligands must mostly be produced on-site. After an intravenous injection, the PET tracer enters the CNS, binds to its corresponding target and can be detected using the PET camera. PET imaging is a non-invasive imaging technique with high molecular sensitivity and specificity, which allows remarkably accurate *in vivo* quantification of the molecules of interest within the CNS (15–17). PET can be highly specific for a disease-related process, provided that a suitable PET tracer is available (18). PET imaging has been so far relatively underused in the evaluation of the disease pathogenesis in MS, despite the potential to be able to detect the pathogenic determinants related to MS pathogenesis *in vivo* and longitudinally in a given individual patient. Here, the detection of activated microglial cells in the context of progressive MS has been the main target of our PET imaging studies (19, 20).

THE TSPO-MOLECULE IS UPREGULATED UPON ACTIVATION OF MICROGLIA

For visualization of microglial activation, radioligands binding to the TSPO molecule are mostly used. TSPO is a protein structure, which is expressed on the outer mitochondrial membrane of activated microglia, and TSPO upregulation on microglial cells is thus considered to be a sensitive "real-time" marker of activation of these cells (21–23). TSPO is also expressed widely outside the CNS and it is thought to be involved in a range of vital cellular functions including regulation of cell proliferation, programmed cell death, steroid biosynthesis, and heme synthesis (24, 25). TSPO also plays a role in cell activation and in opening of the mitochondrial permeability transition pore (26). It was previously called the "peripheral benzodiazepine receptor" (27).

In the "resting" or surveying microglia, TSPO is expressed at a lower level; mainly in the gray matter (28). In non-neoplastic CNS damage without BBB breakdown, microglia are the main cell population expressing TSPO, but also blood-derived macrophages, reactive astrocytes, and endothelial and smooth muscle cells in the vasculature express TSPO (21, 29–33). Interestingly, knocking out TSPO is protective in a mouse model of MS (34). On the other hand, recent *in vitro* work investigated TSPO expression in activated macrophages and surprisingly, a consistent downregulation of TSPO mRNA and protein in macrophages activated to a pro-inflammatory, or "M1" phenotype was demonstrated (35). On the other hand, stimulation of macrophages to an M2 phenotype with IL-4, dexamethasone or TGF-β1 did not alter TSPO expression (35). The same group investigated TSPO expression in rodent vs. human-derived macrophages and microglia upon pro-inflammatory stimulation (36). Here, they demonstrated a ninefold increase in TSPO in rodent-derived macrophages and microglia upon pro-inflammatory stimulation, but surprisingly, TSPO expression did not increase with classical pro-inflammatory activation in primary human microglia. Pro-inflammatory activation of human monocyte-derived macrophages was associated with a reduction of both TSPO gene expression and TSPO-binding site availability. How these *in vitro* experiments relate to MS immunopathology in MS brain *in situ* remains to be seen, but the findings do suggest that changes in TSPO expression in PET imaging studies of MS may reflect microglial and macrophage density rather than activation phenotype (36). Neuropathological studies of TSPO localization in various types and various patho-anatomical locations in MS brain tissue *in situ* are, unfortunately, still relatively limited (21).

RADIOLIGANDS USED FOR DETECTION OF TSPO

First Generation TSPO Ligand [^{11}C] PK11195

The first TSPO-binding compound, PK11195, has been available for more than 30 years (37). [^{11}C]PK11195 was first used for imaging of human gliomas in 1989 (38), and the first *in vivo* human MS brain study was performed in 1997 (39). [^{11}C]PK11195 has high specificity for TSPO (40), but a short half-life (20 min) and low signal-to-noise ratio complicates image analysis (41). [^{11}C] PK11195, like other TSPO-ligands, binds to endothelial cells and to plasma proteins, which needs to be accounted for when evaluating the images. Quantification of specific radioligand binding in a given region of interest (ROI) usually requires comparison to a reference area devoid of specific binding. MS brain naturally lacks such an anatomically clearly defined reference region, which necessitates mathematical modeling of the signal to allow reliable estimation of specific binding to cells of the innate immune system (42–44). For quantification of specific [^{11}C]PK11195 ligand binding, a semi-automated model (supervised clustering algorithm) has been validated (43, 44) and applied in several [^{11}C]PK11195-PET studies of MS (20, 45, 46). Up to date, 12 different studies in MS using [^{11}C]PK11195 have been published (**Table 1**). These studies have evaluated the presence of activated microglia in various cohorts of MS. They have also been used as a prognostic marker for worsening of the disease, or used for measuring the treatment effect of various MS treatments, as discussed below.

Second-Generation TSPO Ligands

Second-generation TSPO ligands with higher affinity and specificity have been developed (23, 54), and over 80 high-affinity TSPO tracers are currently at some stage of development (55). Of these, [^{11}C]PBR28, [^{18}F]PBR111, [^{11}C]FEDAA1106, and [^{18}F] GE180 have already been used in studies of MS (56–60) (**Table 2**). The first studies with these tracers did not show differences in ligand uptake between MS patients and healthy controls (58, 61). However, this was before discovering that in humans the binding affinity for these second-generation ligands is individually determined by genetic variation in the TSPO gene. Thereafter, identification of a single nucleotide polymorphism (rs6971) in exon 4 of TSPO gene has enabled stratification of study subjects into high, medium, and low affinity binders (62), and thus, more accurate estimation of the ligand binding properties is possible at group level (**Table 2**).

Despite the advances in genetic testing, other challenges remain in the image analyses and in estimation of the specific binding of these ligands. As with [^{11}C]PK11195, some of the specific TSPO binding of second-generation ligands appears to be accounted for by binding to activated astrocytes (68) and endothelial cells (69). In addition, the methodology for individual normalization or the choice of a reference region, presumably free of specific binding, is very varied among the human brain studies using second-generation ligands. For example, use of white matter (60) and caudate nucleus (56, 57) as pseudoreference regions as well as whole brain normalization (70) have been reported but not thoroughly validated for [^{11}C]PBR28. In contrast, [^{18}F]GE180 appears to have surprisingly low brain uptake in healthy controls (71), which makes the quantification of specific binding even more challenging, although the methodology for total distribution volume estimation appears feasible (72).

TSPO-PET IMAGING FINDINGS IN DIFFERENT SUBTYPES OF MS

TSPO-PET Imaging in Progressive MS

Studies of progressive MS have demonstrated an increase in TSPO uptake in the NAWM and NAGM which appears to be related to disease severity and patient age (60). In the NAWM of SPMS patients, the TSPO binding is significantly increased when compared to age-matched healthy controls (20, 30, 46, 47, 60). In PPMS, such studies are still lacking. In addition to quantification of the diffuse microglial activation in the NAWM and NAGM, PET imaging can also be used to differentiate between chronic active (smoldering) and chronic inactive lesions. In particular, the slowly expanding/smoldering lesions are thought to contribute to progression of MS and being able to detect these *in vivo*, and to evaluate the kinetics of the plaque evolution *in vivo*, will likely give new information into the pathology driving the progression.

TABLE 1 | Human *in vivo* positron emission tomography (PET) imaging studies with first generation TSPO ligand [^{11}C]PK11195 in multiple sclerosis.

Reference	Study population (*n*)	Main findings on the radioligand binding		
		Lesion associated ROIs	**NAWM/NAGM/other**	**Association with clinical parameters and/or with longitudinal outcome**
Vowinckel et al. (39)	MS (2)	– Increased uptake in a resolving acute WM lesion – Low uptake in chronic T1 lesions	N/A	N/A
Banati et al. (30)	HC (8) RRMS (8) SPMS (1) PPMS (3)	– Higher uptake in 30% of Gd+ than Gd- lesions – Higher mean uptake in T1 black holes/hypointense lesions in RRMS patients during a relapse than without relapse – 1 SPMS patient with higher uptake in T1-hypointense lesions compared to RRMS	– Higher mean uptake in thalami and brainstem of MS vs. HC – Higher hemispheric percentage of voxels with increased (>2SD) binding in 4 patients compared to HC	– Association of higher percentage of TSPO-binding T1 lesion to higher EDSS
Debruyne et al. (47)	HC (7) RRMS (13) SPMS (7) PPMS (2)	– Increased uptake in Gd+ active lesions – Uptake in T2 lesions increased at the time of relapse	– No significant differences in NAWM and GM uptake between HC and all MS patients	– Higher NAWM uptake associated with longer disease duration
Versijpt et al. (48)	HC (8) RRMS (13) SPMS (7) PPMS (2)	– Lower uptake in T2 lesions associated with higher brain atrophy index[a]	– Higher uptake in NAWM associated with higher brain atrophy index[a]	N/A
Ratchford et al. (49)	RRMS (9)	N/A	– Decrease in global cortical GM and cerebral WM uptake after 1 year of treatment with glatiramer acetate	– Decrease in global cortical GM and cerebral WM uptake after 1 year of treatment with glatiramer acetate
Politis et al. (46)	HC (8) RRMS (10) SPMS (8)	N/A	– Higher cortical uptake in MS vs. HC and in wider areas in SPMS vs. RRMS – Higher uptake in WM of SPMS and RRMS vs. HC	– Total cortical binding correlated with EDSS, stronger association in SPMS than in RRMS – No association between Wm binding and clinical disability
Giannetti et al. (45)	RRMS (10) PMS (9; of which 8 SPMS, 1 PPMS)	– Heterogeneity in uptake within T1 black holes, 76% of black holes positive for [^{11}C]PK11195 binding. No difference in distribution between RRMS and PMS – Uptake in [^{11}C]PK11195 positive T1 black holes higher in PMS vs. RRMS	N/A	– Higher uptake in T1 black holes correlates with higher EDSS score in PMS but not in RRMS – Total binding in T1 black holes was a significant disability predictor in PMS at 2 years after TSPO-imaging
Rissanen et al. (20)	HC (8) SPMS (10)	– Increased perilesional uptake in 57% of T1-hypointense lesions – Mean uptake in T2 lesional area lower compared to NAWM in SPMS	– Higher uptake in NAWM and thalami in SPMS vs. HC	N/A
Giannetti et al. (50)	HC (8) CIS (18)	N/A	– Uptake in NAWM higher in CIS than in HC – Mean uptake in NAWM higher in patients with T2 lesions than without – Higher binding in deep but not in cortical GM in CIS vs. HC	– Higher uptake in NAWM correlated to higher EDSS – CIS subjects who developed CDMS by 2 years follow-up had higher uptake in NAWM at baseline
Tarkkonen et al. (51)	RRMS (1)	– Slightly but insignificantly increased [^{11}C] PK11195 binding in a grade II glioma (astrocytoma) when compared to NAWM in a patient with RRMS	N/A	– Moderate uptake of [^{11}C]methionine and non-specific uptake of [^{11}C]PK11195. – Differentiation between a tumefactive demyelinating lesion and low-grade glioma not possible with PET in this case; biopsy confirmed the diagnosis
Sucksdorff et al. (52)	RRMS (11) HC (8)	– No significant difference in mean T2 lesional uptake compared to NAWM in baseline	– Higher uptake in combined NAWM+NAGM ROI and in thalami in RRMS vs. HC in baseline	– Decrease in mean T2 lesional uptake in group level after 6 mo. treatment with fingolimod
Kaunzner et al. (53)	RRMS (16) SPMS (2) HC (6)	– Significantly higher uptake in Gd+ and non-significant trend for higher uptake in Gd- lesions in MS patients compared to normal WM in HC at baseline	– No difference in cortical GM and thalamic binding among MS vs. HC at baseline – Good test-retest reproducibility in HC	– Decreased uptake in individual Gd+ lesions and decreased overall uptake in Gd- lesions at group level after 6 mo. treatment with natalizumab – No longitudinal changes in NAWM or NAGM

TSPO, 18 kDa translocator protein; HC, healthy control; RRMS, relapsing remitting multiple sclerosis; SPMS, secondary progressive multiple sclerosis; PPMS, primary progressive multiple sclerosis; PMS, progressive multiple sclerosis; CIS, clinically isolated syndrome; ROI, region of interest; NAWM, normal-appearing white matter; WM, white matter; Gd+, gadolinium enhancing; Gd–, non-enhancing; EDSS, expanded disability status scale; CDMS, clinically definite MS.

[a]*Brain atrophy index defined as the relative CSF volume divided by the relative white and gray matter volume.*

We found that in the brain of advanced SPMS patients, 57% of the plaques were of the chronic active type, with increased TSPO-binding at the plaque edge demonstrating persisting inflammatory activity in these "holes" (20). **Figure 1** demonstrates a TSPO-PET image with both chronic active and chronic inactive lesions. Similarly, Giannetti et al. demonstrated heterogeneity in [^{11}C]PK11195 binding pattern in black holes (45). Findings from MS studies using later generation TSPO ligands were also in accordance with the above described findings (66, 72).

TSPO-PET Imaging in RRMS

In vivo TSPO-PET imaging has revealed modest microglial activation in the NAWM of RRMS patients, when compared to SPMS (46). Similarly, in neuropathological studies, the diffuse microglial activation outside focal lesions was a feature of progressive disease and was less significant in RRMS patients (7). However, CIS patients who later developed clinically definite MS were shown to have increased TSPO radioligand binding in the NAWM (50). Similarly, during a washout period for a switch in disease modifying therapy, RRMS patients had increased TSPO binding in the NAWM when compared to healthy controls (52). TSPO binding is increased in acute lesions, and T2 lesions have higher TSPO binding during a relapse than during stable disease (39, 48, 53).

TSPO-PET IMAGING AS A PROGNOSTIC MARKER FOR MS WORSENING

Usability of TSPO-PET as a prognostic marker for MS evolution has already been addressed in several studies. Datta et al. found that greater binding of the second-generation TSPO radioligand [^{11}C]PBR28 in the NAWM correlated with subsequently greater MRI activity (enlarging T2 lesion volume) among RRMS patients, and with a greater rate of brain volume loss among patients with SPMS (57). This indirectly suggests that the more substantial total inflammatory burden measured using TSPO-PET might predict faster subsequent progression as both enlarging lesions and the brain atrophy rate have prognostic significance for disability progression in MS (73). Another study demonstrated that an adverse clinical outcome in a group of MS patients correlated with increased TSPO binding at baseline (50). Here, a group of patients converting from clinically isolated syndrome (CIS) to clinically definite MS during a follow-up period of 2 years had higher TSPO binding in the NAWM at baseline compared to the group who retained their CIS status (50). Similarly, those SPMS patients whose EDSS improved over a follow-up period of 30 months had lower TSPO-binding in black holes at baseline compared to patients with worsening EDSS (45).

EFFECT OF MS THERAPEUTICS ON MICROGLIAL ACTIVATION MEASURED USING TSPO-PET

The greatest potential for TSPO-PET imaging over conventional MRI lies in its ability to detect the diffuse compartmentalized inflammation related to microglial activation, and there are expectations for the usability of PET imaging in the quantification of treatment effects of MS drugs targeting microglial activation. The two published longitudinal TSPO-PET studies evaluating microglial activation in the NAWM of MS are by Ratchford et al. (49), and Sucksdorff et al. (52). In the first study, RRMS patients were evaluated before and after 1 year of glatiramer acetate treatment. The study demonstrated that treatment of RRMS with glatiramer acetate reduced TSPO binding significantly in both cortical GM and cerebral WM when using cerebellum as a reference region. The TSPO-PET study by Sucksdorff et al. included three serial PET images of MS patients. After 6 months of fingolimod treatment no statistically significant reduction in microglial activation could be observed in the NAWM or NAGM in the group of ten individuals taking part in the study. A reduction in microglial activation was observed, however, in T2 lesion areas. Similarly, treatment of a focal lesion in a rat EAE model demonstrated a clear reduction in microglial activation after fingolimod treatment (74). The study by Kaunzner et al. demonstrated reduction in microglial activation in focal inflammatory lesions after natalizumab treatment (53). None of these studies included a prospectively followed MS control group without treatment, and a longitudinal study which would evaluate alteration in microglial activation in untreated MS patients over time is still awaited. In fact, longitudinal TSPO-imaging studies are scarce overall. Kreisl et al. reported recently an increase in TSPO binding among patients with Alzheimer's disease over a period of 2.4 years, compared to healthy controls (75). **Tables 1** and **2** list all known MS studies performed so far using TSPO imaging.

FUTURE DIRECTIONS IN PET IMAGING OF ACTIVATED MICROGLIA IN MS

Despite the established role for TSPO-PET imaging in detecting activated microglia *in vivo* there remain challenges. One is that it is presently not possible to differentiate the anti-inflammatory (M2-type) and pro-inflammatory (M1-type) phenotypes of microglia with TSPO targeting radioligands (76). To date, two radioligands targeting the P2X7 purinergic receptor, namely [^{11}C]GSK1482160 (77, 78) and [^{18}F]EFB (79), have been developed and tested in animal models of neuroinflammation. Importantly, the expression of P2X7 in microglia has been associated with a pro-inflammatory M1-like phenotype of these cells (80). If further studies with the P2X7-binding radioligands show potential for their use in humans, they could be applied as imaging biomarkers in future longitudinal observational and treatment studies of neuroinflammatory and neurodegenerative conditions.

Several other targets for PET imaging of microglia have also been proposed, including inducible nitric oxide synthase (iNOS), folate receptor β (FRβ), indoleamine 2,3-dioxygenase-1 (IDO-1), kynurenine-3-monooxygenase (KMO), and cannabinoid receptor 2 (CB2) (81, 82). Of these, iNOS and FRβ may have additional value over TSPO, since iNOS is potentially specific for M1-type

TABLE 2 | Human *in vivo* positron emission tomography (PET) imaging studies with second-generation TSPO ligands in multiple sclerosis.

TSPO ligand	Reference	Study population (*n*)	Main findings on the radioligand binding		
			Lesion associated ROIs	NAWM/NAGM/other	Association with clinical parameters and/or with longitudinal outcome
[^{11}C] vinpocetine and [^{11}C] PK11195	Vas et al. (63)[a]	MS (4; mainly[c] RRMS)	– Lesional and perilesional binding of [^{11}C]vinpocetine higher than with [^{11}C] PK11195 but with low overlap in areas of high uptake between ligands	– Global uptake of [^{11}C]vinpocetine higher than with [^{11}C]PK11195	N/A
[^{11}C]PBR28	Oh et al. (61)[a]	HC (7) MS (11; mainly[c] RRMS)	– Increased uptake in Gd+ WM lesions – Varyingly increased perilesional binding in 71% of T1 lesions – Focally increased uptake in areas preceding development of Gd+ lesions[b]	– No difference in global uptake in MS vs. HC – Higher WM/GM binding ratio in MS vs. HC	– Correlation of higher global binding with longer disease duration, but not with EDSS or MSFC
	Park et al. (64)	HC (4) RRMS (4)	– No difference in T1 lesional vs. NAWM uptake in RRMS patients	– No differences in whole brain GM, whole brain NAWM or regional uptake between MS and HC – Good test–retest reproducibility – Significantly higher SUV but not VT in HABs vs. MABs	N/A
	Datta et al. (65)	RRMS (16) SPMS (7)	– No association between [^{11}C]PBR28 uptake and MRS myo-inositol signal in WM lesions among all patients – Moderate correlation between creatine normalized NAA concentration and [^{11}C] PBR28 uptake in WM lesions	– No association between [^{11}C]PBR28 uptake and MRS [myo-inositol] in NAWM or GM among all patients – Correlation between higher normalized [myo-inositol] and higher [^{11}C]PBR28 binding weighted by WM lesion fraction within patients with high [^{11}C]PBR28 binding	– No association between clinical disability and [^{11}C]PBR28 binding
	Datta et al. (56)[d]	HC (20) RRMS (17) SPMS (7)	– Heterogeneous patterns of binding in WM lesions – Mean uptake in WM lesions lower when compared to NAWM – Higher proportion of inactive lesions in SPMS vs. RRMS	– Higher uptake in NAWM and thalami in MS vs. HC – Strong positive correlation between median WM lesional and NAWM binding	– Higher proportion of inactive lesions in patients with longer disease duration
	Datta et al. (57)	RRMS (14) SPMS (7)	– [^{11}C]PBR28 uptake in WM lesions correlated positively with baseline T2 lesion volume	– [^{11}C]PBR28 uptake in NAWM correlated positively with baseline T2 lesion volume – Negative correlation between MTR in NAWM and [^{11}C]PBR28 uptake in NAWM in baseline	– Enlarging T2 lesion volumes at 1 year follow-up correlated with higher NAWM and WM lesional [^{11}C]PBR28 uptake in baseline in RRMS but not in SPMS – Higher whole brain and GM atrophy rate at 1-year follow-up correlated with higher WM lesional uptake in baseline in SPMS – Non-significant trend for correlation between higher whole brain atrophy rate at 1-year follow-up and higher NAWM uptake in baseline
[^{18}F]PBR111	Colasanti et al. (66)	HC (11) RRMS (11)	– Higher uptake in T2 lesional and perilesional WM in RRMS vs. normal WM in HC group	– Non-significant trend for lower whole WM binding in HC vs. RRMS	– Positive correlation between higher lesional-to-nonlesional WM binding ratio and MS severity scores in RRMS
	Colasanti et al. (67)	HC (22) RRMS (11)	N/A	– Higher hippocampal uptake in RRMS vs. HC – No difference in thalamic uptake in RRMS vs. HC	– Positive correlation of higher hippocampal uptake to higher BDI score in RRMS – Higher age associated with higher hippocampal uptake

(*Continued*)

TABLE 2 | Continued

TSPO ligand	Reference	Study population (*n*)	Main findings on the radioligand binding		
			Lesion associated ROIs	**NAWM/NAGM/other**	**Association with clinical parameters and/or with longitudinal outcome**
	Datta et al.[d] (56)	HC (10) RRMS (10)	– Heterogeneous patterns of binding in WM lesions – No significant difference in lesional vs. NAMW uptake – Higher proportion of inactive lesions in SPMS vs. RRMS	– Higher uptake in NAWM in MS vs. HC – Strong positive correlation between median WM lesional and NAWM binding	– Higher proportion of inactive lesions in patients with longer disease duration
[^{18}F]GE180	Vomacka et al. (59)	HC (6) RRMS (17)	– Increased mean uptake in MS lesions	– Higher uptake in WM and thalami in RRMS vs. HC	N/A
[^{11}C] FEDAA1106	Takano et al. (58)[a]	HC (5) RRMS (9)	– Reliable lesional binding estimates not obtainable due to noisy time activity curves – High uptake in one Gd+ lesion in one patient	– No difference in global or regional uptake between RRMS and HC	N/A

TSPO, 18 kDa translocator protein; HC, healthy control; RRMS, relapsing remitting multiple sclerosis; SPMS, secondary progressive multiple sclerosis; PPMS, primary progressive multiple sclerosis; PMS, progressive multiple sclerosis; CIS, clinically isolated syndrome; ROI, region of interest; NAWM, normal-appearing white matter; WM, white matter; Gd+, gadolinium enhancing; Gd–, non-enhancing; EDSS, expanded disability status scale; HAB, high-affinity binder; MAB, mixed-affinity binder; MRS, magnetic resonance spectroscopy; NAA, N-acetyl aspartate; BDI, Beck Depression Inventory; MTR, magnetization transfer ratio.

[a]*No genotyping for the single nucleotide polymorphism (rs6971) in TSPO gene affecting the radioligand binding affinity, which possibly affects the interpretation of results.*

[b]*Follow-up MRI performed at 1 month after PET imaging for some of the MS patients.*

[c]*Exact disease type not reported; longitudinal PET data reported only for one patient.*

[d]*Study reporting findings from both [^{11}C]PBR28 and [^{18}F]PBR111, results from both ligands pooled in the lesion binding characterization.*

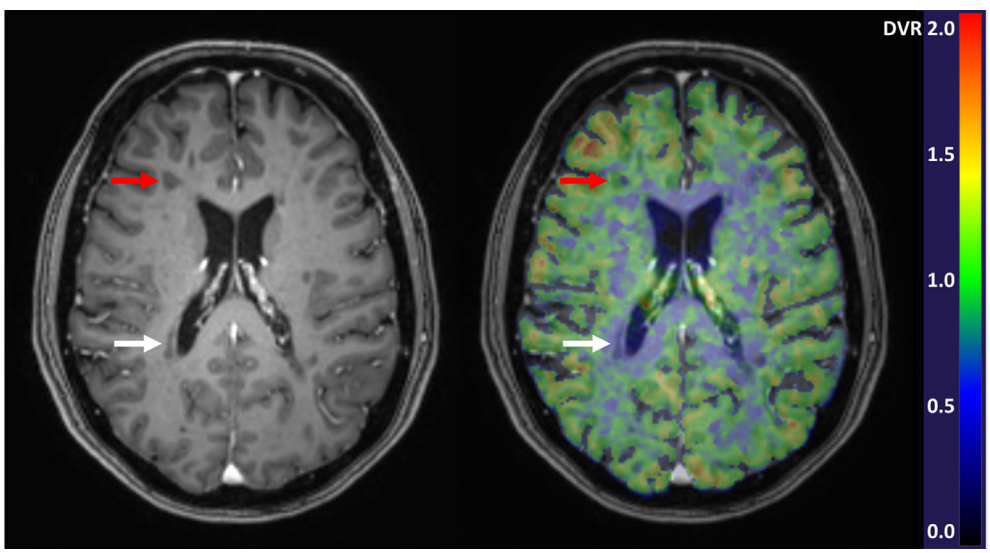

FIGURE 1 | Gadolinium enhanced 3DT1 MRI image (left) and parametric [^{11}C]PK11195-PET image overlayed with the 3DT1 image (right). Red arrows point to a chronic active T1-hypointense lesion with increased perilesional [^{11}C]PK11195 binding demonstrative of microglial activation, and white arrows point to a chronic inactive lesion with negligible radioligand binding. In the parametric PET image, the color of each voxel represents the intensity of specific radioligand binding measured as distribution volume ratio (DVR) and denoted by the scaled color bar.

pro-inflammatory cells, and FRβ for the M2-type homeostatic phenotype of microglia (83, 84). Radioligands for KMO have not yet been developed, and radiotracers for IDO-1 and FRβ have so far been use only in preclinical studies (85–88). Several ligands for CB2 have been developed and tested, but none of these have been found to be suitable for clinical use (82). The first human dosimetry study (89) and one pulmonary imaging study with an endotoxin challenge in healthy subjects using the iNOS-binding radioligand [^{18}F]NOS have been reported (90), but no brain imaging studies with this radioligand have been published. However, pitfall of using iNOS-binding radioligands in the estimation of brain microglial activation is that iNOS is expressed also in macrophages and astrocytes, in addition to microglia (91).

CONCLUSION

Detection of microglial activation in MS brain using *in vivo* PET imaging has already increased our understanding of MS pathogenesis. In the future, we can expect PET imaging to provide alternative methods to monitor the disease progression, to improve the evaluation of therapeutic needs, particularly in progressive MS, and to help choose MS patients most at risk for progression into therapeutic trials of progressive MS. TSPO-PET could also be used as an important surrogate marker in therapeutic studies of progressive MS. There are still technical challenges, such as the poor signal-to noise ratio of the [^{11}C] PK11195 radioligand, and the genetically determined variation in the binding affinity for the second-generation tracers. Moreover, heterogeneity in TSPO image analysis methodology across different imaging centers makes it difficult to perform direct comparisons between the studies. It will be important to harmonize and validate the methodology used in TSPO-PET imaging to allow multi-center studies for evaluation of larger patient cohorts. The great expense and the high technical requirements of nuclear medicine make PET a demanding technology. Nonetheless, the potential of PET imaging to visualize hidden inflammation and other pathogenic determinants in MS brain *in vivo* makes the pursuit of development of yet better ligands a worthwhile effort.

AUTHOR CONTRIBUTIONS

Drafting of the manuscript and critical revision of the manuscript: LA, MN, and ER. Study supervision: LA.

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REVIEW
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The Inflammatory Continuum of Traumatic Brain Injury and Alzheimer's Disease

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The post-injury inflammatory response is a key mediator in long-term recovery from traumatic brain injury (TBI). Moreover, the immune response to TBI, mediated by microglia and macrophages, is influenced by existing brain pathology and by secondary immune challenges. For example, recent evidence shows that the presence of beta-amyloid and phosphorylated tau protein, two hallmark features of AD that increase during normal aging, substantially alter the macrophage response to TBI. Additional data demonstrate that post-injury microglia are "primed" and become hyper-reactive following a subsequent acute immune challenge thereby worsening recovery. These alterations may increase the incidence of neuropsychiatric complications after TBI and may also increase the frequency of neurodegenerative pathology. Therefore, the purpose of this review is to summarize experimental studies examining the relationship between TBI and development of AD-like pathology with an emphasis on the acute and chronic microglial and macrophage response following injury. Furthermore, studies will be highlighted that examine the degree to which beta-amyloid and tau accumulation as well as pre- and post-injury immune stressors influence outcome after TBI. Collectively, the studies described in this review suggest that the brain's immune response to injury is a key mediator in recovery, and if compromised by previous, coincident, or subsequent immune stressors, post-injury pathology and behavioral recovery will be altered.

Keywords: traumatic brain injury, Alzheimer's disease, neuroinflammation, microglia, monocyte, macrophage

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INTRODUCTION TO TRAUMATIC BRAIN INJURY (TBI) AND ALZHEIMER'S DISEASE (AD)

Traumatic brain injury is a significant health concern affecting millions of individuals worldwide. Within the United States (U.S.), the Centers for Disease Control and Prevention report that 1.7 million individuals sustain a TBI annually, and 5.3 million individuals live with TBI-related disabilities (1). Similar structured reporting is limited from the rest of the world. Nonetheless, systematic reviews indicate that more than 7.7 million individuals live with TBI-related disabilities in the European Union (2). Subsequent reviews indicate that increased motor vehicle use is associated with a rising incidence of TBI globally (2–4). A significant concern is that standardized reporting and categorization in epidemiological studies around the world is absent. Consequently, TBI has been described as a "silent epidemic" for multiple reasons. First, epidemiological reports likely reflect an underestimation of incidence, particularly for milder forms of brain injury. Second, without an accurate incidence rate, it is impossible to identify the true public health and economic consequence

of brain injury, including caregiver burden. Third, survivors of mild to moderate brain injury often display delayed and task-specific impairments making chronic, time-dependent reporting essential in documenting long-term effects of TBI. Finally, many post-injury problems are not visible, including cognitive and emotional impairment. Together, these points emphasize the many challenges that we face in attempting to improve recovery following TBI.

Age is closely associated with the incidence of TBI and likely plays a critical role in mediating response to and recovery from brain injury. For example, in the U.S. children aged 0–4 years, adolescents aged 15–19 years, and adults aged 65 years and older are among the most likely to sustain a TBI. Post-injury hospitalization and death are most common in adults aged 75 years and older, suggesting that age at the time of injury and aging after injury are important mediators of long-term recovery. Although a TBI occurs in a matter of milliseconds, the biological consequences of a brain injury may last a lifetime. Indeed, TBI is recognized as an environmental risk factor for many neurodegenerative diseases such as AD, Parkinson's disease (PD), and chronic traumatic encephalopathy (CTE). The molecular mechanisms that link TBI to development of neurodegenerative disease remain underexplored and few studies account for age-specific pathological response to and recovery from brain injury.

Alzheimer's disease is a neurodegenerative disease that progresses from mild cognitive impairment to severe dementia over time (5). The disease is characterized by key neuropathological features, including extracellular accumulation of beta-amyloid (Aβ) protein in senile plaques (6) and intracellular aggregation of microtubule-associated protein tau (MAPT, tau) in neurofibrillary tangles (NFTs) (7). Importantly, both amyloid and neurofibrillary changes begin during preclinical AD when cognitive deficits are not apparent (8). In typical cases of AD, Aβ spreads from the frontal and temporal lobes to the hippocampus and limbic system. NFTs spread from the medial temporal lobes and hippocampus to the neocortex (9). Several lines of evidence point to a relationship between single-incident TBI and AD (10, 11). First, numerous population-based studies demonstrate that head injury during adulthood increases the risk of AD later in life (12–19) and reduces the time to onset of AD (20). Second, many animal studies show increased production and accumulation of amyloid precursor protein (APP), Aβ, and pathological tau following TBI (16, 21–28). Third, accumulation of APP and extracellular deposition of the 40- to 42-amino acid Aβ peptide in senile plaques has been identified in human brain tissue soon after severe TBI (29, 30). Fourth, a comprehensive immunohistochemical study by Ikonomovic and colleagues identifies increased neuronal APP and diffuse Aβ deposits along with diffuse tau immunostaining in neuronal cell somata and axons, as well as glial cells, in resected temporal cortical brain tissue after TBI (31). Intracellular aggregation of MAPT in NFTs was only present in a subset of older subjects (31). These and other studies indicate that there is a biological link between TBI and AD pathology, but the exact molecular pathways underlying this relationship are poorly understood and post-injury mechanisms that facilitate Aβ and tau pathology remain under investigation. This review will consider post-injury neuroinflammation as a malleable response that is closely associated with development of AD-like pathology, thereby supporting a relationship between TBI, neuroinflammation, and development of AD.

A longstanding concern with many clinical studies is reliance on self-report and use of diagnostic verbiage in medical records to identify a correlation between TBI and post-injury development of AD (32). Also, several clinical studies report that individuals with genetic predisposition to developing AD (ApoE4 risk alleles) display altered outcome after TBI making the distinction between environmental and genetic risk factors for post-injury recovery unclear (33, 34). Despite preclinical studies providing evidence for successful pharmacologic intervention, more than 30 phase-III clinical trials have failed to improve secondary injury outcome measures after TBI (35–37). Finally, several experimental studies have failed to show that TBI induces or worsens AD-related pathology (38, 39) with some even reporting a reduction in post-injury Aβ accumulation in transgenic mice (40, 41). Collectively, these results highlight the complex nature of TBI and emphasize the need to clearly define post-injury mediating factors that could be contributing to variability in experimental and clinical studies.

Experimental Models of TBI

To date, no effective interventions are available to improve recovery following TBI (42–44). Thus, experimental models are, therefore, essential in better understanding post-injury pathology and identifying effective therapeutic treatments. This strategy presents additional challenges as each animal model reflects a specific type of TBI and does not fully recapitulate primary and secondary damage evident in human TBI (45) resulting in restricted translation (46). Nonetheless, experimental models represent a critical tool in defining precise mechanisms of primary and secondary damage following TBI, particularly when the data generated are evaluated within the context of the injury model used.

A variety of TBI models are used in experimental studies. While no single model recapitulates all aspects of human TBI, the neuroinflammatory response to injury occurs in a temporally distinct manner. Several excellent reviews are already available that describe contemporary experimental models of injury as well as the inflammatory response to injury [see reviews in Ref. (47–49)]. Here, we will provide a brief description of the models described in this review. Experimental models of TBI have historically been referred to as focal or diffuse, but increasing evidence indicates that even focal brain injuries cause diffuse damage that is not restricted to the site of injury. In addition, concussive, repetitive, and blast-related TBI are often defined as diffuse injuries; however, collectively referring to them as "diffuse" undervalues the variability of the primary insult. Thus, describing the key neuropathological features is a more appropriate strategy for distinguishing experimental models from one another (49). Focal contusion models include controlled cortical impact (CCI) (50), fluid percussion injury (FPI) (51, 52), weight drop (53, 54), penetrating ballistic-brain injury (PBBI) (55). As implied, an external force (impactor tip, fluid, weight, and inflatable probe, respectively) is used to induce TBI and can be manipulated to produce a mild, moderate, or severe brain injury (as defined by post-injury pathology). Predominant pathological

features include a focal contusion, blood–brain barrier (BBB) disruption, edema, in addition to neuronal and axonal damage. There is a widespread inflammatory response, including microglial and astrocytic activation, infiltration or peripheral cells, and increased production and release of inflammatory molecules which are reported up to 1 year post-injury (56). Recently, modified versions of the CCI without craniectomy have been employed to study single and repetitive forms of TBI (57, 58). In studies examining single or multiple mild severity TBI, skull fracture and cortical contusion are absent but neuroinflammation and behavioral impairment persist with increasing number of injuries (57). Studies discussed in this review include 2–30 injuries, with 30 injuries considered a highly repetitive model of TBI (59). In studies examining severe TBI, skull fracture and cortical contusion are induced *via* electrical weight drop on the exposed skull [closed head injury (CHI) model (60)]. Cryogenic brain injury is created when a cotton tip applicator dipped in liquid nitrogen is pressed on top of the skull. This type of injury does not directly induce cortical contusion but facilities an inflammatory response (61). Finally, a chronic hippocampal lesion model of brain injury will be discussed to highlight the effects of microglial elimination in post-injury outcome. The tetracycline-inducible promoter system is used to regulate neuronal expression of diphtheria toxin A-chain in this transgenic mouse model of injury. As a result, forebrain neurons expressing calcium-calmodulin kinas II α (CaMKIIα) are ablated resulting in neuronal loss, inflammation, and behavioral impairment (62).

In summary, these models of experimental TBI induce a temporal inflammatory response that is consistent with what is observed in human head injury, and increased injury severity is positively correlated with BBB disruption and infiltration of peripheral cells (63). Inflammatory cytokines and chemokines are immediately release after moderate TBI and peak within hours post-injury. Consequently, peripheral cells, such as neutrophils, monocytes, T-cells, and dendritic cells, enter the brain within days post-injury. Similarly, microglia and astrocyte reactivity increases within days post-injury, but altered and reactive morphology diminishes by 10–14 days post-injury. Chronic microglial and astrocytic reactivity, as defined by altered morphology, persists in sub-cortical brain regions months to years post-injury. Experimental TBI, regardless of model, consistently induces an inflammatory response including microglial/macrophage reactivity. This conserved response is, therefore, viewed as a critical mediator of post-injury outcome.

Inflammation as a Mediator of Post-Injury Outcome

Primary damage occurs as a result of the physical and mechanical forces of brain injury and includes brain contusion, hemorrhage, hematoma, and axonal injury (3). Secondary damage develops hours and days after the primary damage, but is not necessarily dependent on the primary injury itself. In other words, secondary injury cascades can act concurrently and synergize to influence outcome (3). Secondary damage includes excitotoxicity (64), oxidative stress (65), and widespread neuroinflammation (63). Successful management of post-injury recovery is dependent on effectively stratifying these variables to determine which are predictive in outcome. We propose that the brain's immune response to injury is a key mediator in recovery, and if compromised by *previous*, *coincident*, or *subsequent* immune stressors, progressive impairments will be evident.

Inflammation following TBI is a complex and dynamic response of both the central and peripheral nervous systems, which is influenced by age, sex, injury location and severity, secondary injury cascades, and genetics (63). Multiple other reviews eloquently describe this inflammatory process and provide insight into cell types and molecular pathways involved in the response (48, 63, 66, 67). Because inflammation occurs after all brain injuries, some propose that immune modulation is an integral component to identifying effective and clinically relevant therapeutic interventions (68). It is necessary to acknowledge that post-injury inflammation poses both beneficial and detrimental consequences that need to be balanced. A detailed understanding of mechanisms driving immune activation after TBI is, therefore, of utmost importance (67). In this review, we would like to extend the conversation to include appreciation of the inflammatory continuum that occurs over a lifetime. TBI is not an isolated event within the inflammatory milieu. Accumulating data indicate that pre- and post-injury immune challenges may influence the microglial and macrophage response to brain injury and influence post-injury pathology and behavioral recovery.

Relevance of Microglia and Macrophages in TBI

This review will focus on the role of brain-resident microglia and infiltrating peripheral monocytes. In normal physiological conditions, the BBB prevents entry of peripheral monocytes into the brain parenchyma. Disruption and dysfunction of the BBB after TBI facilities monocyte infiltration though. When in the reactive macrophage state, it is difficult to distinguish microglia and peripheral monocytes. For example, after TBI, microglia and monocyte-derived macrophages adopt a similar morphology, upregulate similar inflammatory surface markers, and increase production of similar inflammatory cytokines. Indeed, many prevalent immunohistochemical markers do not identify whether or not a reactive macrophage is of microglial or monocytic origin. Given the distinct genetic and transcriptomic profile of microglia and macrophages identified *via* high throughput sequencing technology (69–71), the two cell types likely maintain different roles in the injured central nervous system (CNS) (72–74).

Targeted genetic deletion of key chemokine receptors, CCR2 and CX3CR1, has emerged as a useful tool to characterize the role of microglia and macrophages following TBI (48). The surface glycoprotein Ly6C can be used in combination with CCR2 and CX3CR1 to identify two distinct populations of monocytes in peripheral blood, $Ly6C^{high}/CX3CR1^{low}/CCR2^{+}$ and $Ly6C^{low}/CX3CR1^{high}/CCR2^{-}$. The former is the inflammatory subset of monocytes that differentiate into inflammatory macrophages in response to post-injury inflammation. The latter is the patrolling subset of monocytes that survey the vasculature and resolve inflammation. CCR2 is required for monocytes to enter the CNS and, therefore, all infiltrating monocytes are $CCR2^{+}$; however, downregulation of CCR2 following CNS entry has been reported. Genetic deletion of CC ligand-2 (CCL2), the

cognate ligand for CCR2, attenuates lesion volume, reduces macrophage recruitment and astrogliosis, and improves functional outcome compared to controls after CHI (60). Similarly, post-injury macrophage recruitment substantially decreases in *Ccr2*$^{-/-}$ mice following CCI TBI and correlates with improved behavioral outcome (75). Separate groups have shown that CCX872 and RS504393, two selective CCR2 antagonists, reduce post-injury macrophage recruitment and improve functional recovery after CCI and weight drop TBI (76, 77). Together these studies indicate that interruption of CCL2/CCR2 signaling offers therapeutic potential to improve outcome following TBI and lends support to the notion that a persistent post-injury macrophage response is detrimental to outcome. Interruption of CCR2 signaling through the use of *Ccr2*$^{RFP/RFP}$ mice reduces post-injury monocytic infiltration and axonal pathology but enhances cortical and hippocampal MAPT mislocalization and hyperphosphorylation soon after lateral fluid percussion TBI suggesting that monocyte sub-populations may differentially influence outcome (78). Without detailed follow-up studies, the roles of monocyte sub-populations in mediating outcome from TBI remain unknown. Collectively, these studies indicate that interruption of post-injury monocytic infiltration has both beneficial and detrimental consequences depending on the outcome measures evaluated.

The microglial response to TBI has been explored *via* genetic manipulation of CX3CR1. For example, fractalkine (CX3CL1) and its cognate receptor CX3CR1 represent a unique one-to-one ligand–receptor pair. In the CNS, CX3CL1 is highly expressed in neurons and CX3CR1 is expressed by microglia from their first entry into the neuroepithelial parenchyma around E10 throughout adulthood (79). Microglia remain uniformly CX3CR1^{+} and do not express CCR2 or downregulate CX3CR1, even during severe neuroinflammation evident after TBI. After a single CCI TBI, *Cx3cr1*$^{-/-}$ mice have improved motor recovery and decreased neuronal loss through 15 days post-injury (DPI). By 30 DPI, however, these *Cx3cr1*$^{-/-}$ mice have worse cognitive dysfunction and neuronal loss compared to wild-type controls. These changes are directly associated with an altered and time-dependent inflammatory profile in microglia (80). Subsequent work by a separate group confirms these results and demonstrates that CX3CR1 deficiency results in early protection but chronic worsening of CCI TBI-induced deficits due in part to a decrease in anti-inflammatory cytokines on CD11b^{+} sorted cells at 28 DPI (81). Together, these studies emphasize the temporal inflammatory response to a single brain injury and confirm that alteration of this response can influence outcome. Moreover, acute transient interruption of the microglial response to TBI is beneficial to outcome.

Use of *CX3CR1*$^{GFP/+}$*CCR2*$^{RFP/+}$ reporter mice provides insight into the role of microglia and macrophages following TBI (76); however, it is still unclear if myeloid cells associated with chronic injury lesions are CX3CR1^{+} microglia or a mixture of CX3CR1^{+} microglia and CX3CR1^{+} peripheral macrophages that downregulated CCR2. Future studies geared to address the therapeutic potential of targeting specific sub-populations of reactive macrophages may hold great translational significance. Because the cell-specific role of microglia and blood-derived macrophages in post-injury recovery remains limited, they will be collectively referred to as macrophages within this review.

POST-INJURY NEUROINFLAMMATION AND AGING

Accumulating evidence implicates the post-injury inflammatory response as a key mediator in long-term recovery from TBI. Many biological pathways are disrupted by experimental TBI resulting in progressive neurodegeneration including atrophy, neuronal loss, and axonal degeneration which are often associated with neuroinflammation including macrophage reactivity (82–85). These findings are consistent with human studies that report increased mRNA expression of microglial markers OX-6 and CD68 at 1 year post-injury (11) and imaging studies showing increased binding of PK-[11C](R)PK11195 ligand, expressed by activated microglia, between 11 months and 17 years post-injury (86). Macrophage-mediated neuroinflammation is also a prominent feature of many age-related neurodegenerative diseases including AD (87). For example, myeloid cells are instrumental in maintaining CNS homeostasis; however, aging significantly alters their properties (88). Consequently, age-related immune changes and those that occur during AD share many similarities and the distinction between the two processes remains unclear (89). Determining the extent to which age-related impairments in myeloid functioning facilitates accumulation of Aβ or if accumulation of Aβ impairs myeloid functioning is critical in identifying which immune pathways should be targeted (90). Moreover, inflammation is a malleable response to TBI that changes with aging which suggests that it could be critical in mediating post-injury outcome.

Age-related changes in the function of microglia and macrophages may influence outcome after TBI. For example, phagocytosis and chemotaxis diminish in both microglia and macrophages during aging. While the age-related pro-inflammatory response to immune challenge is decreased in macrophages (91), aged microglia displayed an exaggerated pro-inflammatory response referred to as "microglial priming" first described in a model of prion disease (92). A primed microglia profile includes (1) increased basal expression of inflammatory markers and mediators, (2) decreased activation threshold to express and release pro-inflammatory molecules, and (3) exaggerated inflammatory response to immune challenge (93). The cause of microglial priming is unclear and likely results from multiple factors, including but not limited to (1) a loss of inhibitory ligand–receptor communication with aging neurons (94), (2) interactions with age-related misfolded proteins such as Aβ which promote pro-inflammatory cytokine production (95), (3) age-related exposure to increasing transforming growth factor-β which could compromise microglial transitioning from a pro- to anti-inflammatory phenotype (96), (4) age-related alterations in production of IL-4 and CCL11 in the choroid plexus (97–99), and (5) unique microenvironment effects in white and gray matter. For example, previous studies demonstrate that gray matter injury elicits an enhanced macrophage response in older rodents compared to younger rodents (100, 101); however, white

matter demyelination injury provokes a reduced macrophage response in older rodents compared to younger controls (102). Collectively, these data indicate that aging before and after TBI could significantly influence outcome.

Taken together, these findings indicate that the immune response to and recovery from TBI is not absolute and very much influenced by multiple factors. Existing brain pathology and secondary immune challenges may be critical in shaping post-injury disease pathogenesis. Indeed, macrophage-mediated inflammation across the continuum of aging should be considered in the context of TBI, particularly when studying outcome related to development of neurodegenerative disease. Therefore, the primary purpose of this review is to summarize studies examining the relationship between single-incident TBI and development of AD-like pathology with an emphasis on the acute and chronic microglia and macrophage response following injury. Repetitive TBI will be considered as a repeated immune stressor and discussed only briefly. Furthermore, studies will be highlighted that examine the degree to which pathological protein accumulation and peripheral immune stressors influence outcome after TBI.

TBI, Inflammation, and AD

Chronic inflammation is a potential common denominator in both TBI and AD. TBI induces a widespread neuroinflammatory response that can promote recovery if controlled for a defined time period. Excessive or chronic neuroinflammation is linked to progressive changes, including atrophy, neuronal loss, and axonal degeneration (84, 103–105). Post-injury neuroinflammation is characterized by activation of brain-resident microglia, infiltration of peripheral immune cells, astrogliosis, and increased synthesis and release of pro- and anti-inflammatory molecules which can persist for months to years after the initial insult (106, 107). There is a persuasive body of evidence showing a significant inflammatory component in AD as well. First, microglia, monocytes, and astrocytes as well as inflammatory cytokines and chemokines are elevated in the AD brain (108). Second, retrospective studies demonstrate that sustained NSAID treatment during mid-life significantly decreases the risk of AD (109, 110). Considering the failure of prospective studies with NSAID treatment (111), the beneficial effects of NSAIDs is presumed to be related to premorbid function. Third, recent genetic studies implicate inflammatory genes and pathways (*CD33, TREM2, HLA-DRB5*-DRB1) in late-onset disease pathology (112–115). Fourth, alterations in inflammatory cells and molecules are reported in multiple different mouse models of AD. Finally, accumulating evidence shows that microglia and monocytes play distinct roles in AD pathogenesis (116–119), thus implicating both the central and peripheral immune response in long-term outcome. Collectively, these results suggest that chronic post-injury neuroinflammation may be sufficient to induce or facilitate AD-related pathology.

TBI AND AMYLOID-RELATED PATHOLOGY

Rodent models have been a valuable resource in studying the relationship between TBI and AD-like pathology [see reviews (10, 120)]; however, most of the early studies focused on accumulation and production of Aβ. Many types of CNS injury, including TBI, induce the expression of APP. For example, APP expression increases in striatal and hippocampal axons along with cortical and thalamic neurons within the first 24 h after experimental impact and fluid percussion TBI (121–123) which has been replicated in multiple follow-up studies using CCI as well as midline and lateral FPI (124–126). Traumatic axonal injury (TAI) is an additional source of accumulating APP (127, 128). For example, APP accumulates in traumatized axons after all severities of TBI and has been detected many months post-injury (129–131).

Amyloid precursor protein accumulation does not result in Aβ deposition in many experimental studies though. Although Aβ deposition is apparent following rotational acceleration TBI in pigs (132, 133) and rabbits (134), a majority of rodent studies fail to show this association in non-transgenic animals using CCI, FPI, and weight drop models (121, 122, 124, 125, 135). Consequently, the validity of TBI-AD experiments in non-transgenic rodents is unclear. Many factors likely contribute to the lack of Aβ deposition in these studies. For example, multiple reports indicate that there are endogenous differences in rodent and human APP (136), which could significantly alter the production on Aβ after TBI. Injury severity may also be a critical mediator in outcome. Clinical studies indicate that Aβ accumulates within hours after severe TBI and is spread throughout the cerebral cortex compared to age-matched controls (29, 31). Indeed, the complex neuroanatomy and neurophysiology of the human brain, such as cortical folding, substantial white matter, and specific pathophysiology compared to the rodent brain, may facilitate distinct post-injury neuropathology (137). Finally, location and timing of injury may mediate Aβ pathology. For example, Aβ accumulation is observed in patients with *dementia pugilistica* which reflects traumatic injury as a result of repetitive brain insults. Thus, Aβ may have a specific temporal profile in single and repetitive models of TBI.

The availability of transgenic and knock-in mouse models of AD expressing wild-type or mutant human APP provided an additional avenue of study to determine the relationship between TBI and amyloid-related pathology. One of the earliest mouse models of AD utilized a platelet-derived growth factor-β promoter to overexpress mutant human APP. These PDAPP transgenic mice display age-related cognitive impairment, synaptic dysfunction, Aβ accumulation, and tau phosphorylation. Although CCI TBI induced a surge of plaque pathology in PDAPP mice soon after injury at 6 months of age, a substantial reduction in cortical and hippocampal plaque load was detected chronically (28, 40). Follow-up experiments revealed that CCI TBI in aged PDAPP mice caused a regression of established Aβ deposits (41). In both sets of experiments, the reduction in Aβ pathology was accompanied by increased neuronal death and memory impairment, ultimately bringing into question the neurotoxic properties of Aβ alone.

Monomeric Aβ aggregates to form oligomers, protofibrils, and fibrils that accumulate in the characteristic Aβ plaque of AD. Thus, the production of Aβ is a complex process and accumulating evidence indicates that soluble Aβ oligomers, not Aβ plaques alone, are the disease-causing species that induce substantial neurotoxicity including synaptic dysfunction and behavioral impairment [see review in Ref. (138)]. Surprisingly, the role of

soluble Aβ oligomers in post-injury pathology has received limited attention. While several clinical studies report that higher cerebral spinal fluid (CSF) levels of Aβ42 predict improved neurological recovery following severe TBI (139, 140), higher levels of CSF Aβ oligomers predict poor neurological recovery (141). A single experimental study examined the accumulation of soluble, insoluble, and oligomeric Aβ following TBI in the 3xTg mouse model of AD which harbors (overexpressed) transgenes carrying genetic mutations that promote Aβ and tau pathology. While CCI TBI increased soluble and insoluble cortical Aβ40 and Aβ42 within 24 h after injury, both soluble and insoluble Aβ returned to sham levels by 7 DPI (23). Although these studies indicate that TBI induces an acute increase in oligomeric Aβ, the long-term consequences of this increase and the effect on specific cell types or brain region pathology remains unknown.

Based on the abovementioned results, one might suggest that the validity of TBI-AD experiments in APP-transgenic rodents is unclear as well. Indeed, many of these models express mutant APP at higher levels than endogenous APP and maintain genetic risk variants that cause familial AD which is fairly uncommon. The co-occurrence of TBI and APP mutation in the clinical setting is rare (29) thereby restricting the results of many of these studies. Recent findings shed light on the discrepancies between experimental TBI-AD studies and emphasize the potential role of non-neuronal cells in mediating outcome. For example, TBI in the APP/PS1 knock-in mouse model of AD results in a delayed neuroinflammatory response compared to wild-type control mice subjected to CHI (16). While both brain-injured AD and wild-type mice had increased expression of inflammatory cytokines IL-1β, IL-6, and TNFα, peak elevations were delayed by 7 days in the AD mice but persisted in conjunction with astrocyte activation. A similar trend was observed in the chemokines CCL2, CCL3, CCL4, and CCL5. In addition, mRNA expression of CCR2, CD68, and MHC-II, characteristically expressed by macrophages, was delayed in APP/PS1 mice compared to wild-type controls following TBI. Treatment with MW151, a small-molecule inhibitor targeting pro-inflammatory cytokines in glia, attenuated the persistent increase in pro-inflammatory cytokine expression and improved cognitive recovery in APP/PS1 mice. Collectively, these results indicate that there is a direct relationship between neuroinflammation and functional recovery and emphasize the distinct temporal inflammatory response to TBI in APP/PS1 mice (16).

The immunomodulatory effects of accumulating Aβ were confirmed in another set of TBI experiments. A separate group of investigators examined the macrophage response to lateral FPI in the R1.40 mouse model of AD, which maintains genetic predisposition to developing Aβ deposits between 12 and 15 months of age *via* multiple copies of the mutant APP yeast artificial chromosome (38). TBI was administered to young, 2-month-old mice to determine if brain injury worsened or advanced the appearance of age-related AD-like pathology. The acute macrophage response to TBI, as measured by Iba1, CD45, F4/80, CD68, and Trem2 immunohistochemistry, was strikingly muted in R1.40 TBI mice compared to wild-type mice exposed to TBI. Flow cytometry revealed that reduced numbers of myeloid cells acquired a macrophage phenotype in R1.40 TBI mice, correlating with decreased inflammatory cytokine expression. At a chronic time point, several months after TBI, the macrophage response to injury subsided in wild-type mice; however, it was relatively unchanged in R1.40 mice. In addition, R1.40 mice displayed significant tissue loss between 3 and 120 DPI and task-specific cognitive deficits in transferring information from 1 day to the next at 120 DPI. Importantly, TBI did not advance the appearance of Aβ plaques in R1.40 mice. Together, these findings emphasize the potential neuromodulatory role of accumulating Aβ and demonstrate that the glial response to TBI is altered in the presence of Aβ and correlates with altered functional recovery (38).

The immunomodulatory role of Aβ has been manipulated in other experimental models. For example, a 2013 study revealed that peripheral administration of Aβ42 and Aβ40 attenuates paralysis and reduces neuroinflammation in multiple mouse models of experimental autoimmune encephalomyelitis (EAE) (142). Aβ specifically suppressed cytokine secretion in activated peripheral lymphocytes and reduced inflammatory foci within the CNS without promoting Aβ deposition in the brain. These results indicate that Aβ maintains both pathological and beneficial properties which are dependent on the type of CNS injury and the inflammatory context, namely lymphoid or brain tissue. Follow-up studies show that a potent hexapeptide core structure in amyloid is highly immunosuppressive and likely mediating these effects to some degree (143). A 2016 study demonstrates that Aβ is anti-microbial and protects against *Salmonella enterica* serotype Typhimurium (*S.* Typhimurium) infection in the 5XFAD transgenic mouse model of AD potentially *via* oligomerization. *S.* Typhimurium infection induced Aβ deposition in 1-month-old 5XFAD mice compared to control 5XFAD mice, which appeared to surround and entrap bacterial colonies (144). The idea of using Aβ as a therapeutic is directly contrary to Aβ strategies in AD, which aim to remove Aβ from the brain. Nonetheless, these studies highlight a physiological role for Aβ in innate immunity and emphasize the effect of Aβ on other cell types which directly influences disease pathogenesis and functional outcome.

What does this mean for experimental TBI-AD research? In fact, the role of Aβ in mediating response to and recovery from TBI is largely unknown and may contribute to the variability in experimental and clinical studies examining the relationship between the two pathologies. For example, many studies report the presence or absence of Aβ as a primary dependent variable of interest following TBI with little attention given to oligomers, protofibrils, and fibrils. Based on recent evidence, the presence of these low-molecular weight aggregates may substantially alter the neuroinflammatory environment and influence outcome following TBI. Given that Aβ alone is not predictive of AD and many older neuropsychologically healthy individuals display amyloid deposition (145, 146), age-related Aβ accumulation may play a critical role in the brain's ability to respond to and recover from traumatic injury. Many techniques are available to identify cell-specific changes following TBI which could be incorporated into future experimental TBI studies. For example, generating AD mice with targeted deletion of CCR2 or CX3CR1 could provide information on the cell-specific response of microglia and monocytes to TBI in the presence of accumulating Aβ. Subsequent fluorescence-activated cell sorting (FACS) would

allow investigators to identify the cell-specific inflammatory profile of microglia and monocytes in this context. In addition, laser capture microdissection of macrophages near and away from Aβ plaques could be useful in identifying the spatial influence of Aβ accumulation. Finally, consistent inclusion of non-transgenic control mice would provide investigators with an opportunity to determine if transgenes of interest influence the response to and recovery from TBI.

TBI AND TAU-RELATED PATHOLOGY

Tau is a scaffolding protein found in neurons and enriched in axons where it regulates microtubule assembly primarily *via* phosphorylation. Increased tau phosphorylation reduces microtubule affinity and supports neuronal plasticity and axonal transport at the synapse (147). Under pathological conditions, such as those occurring in AD, increased post-translational modification of tau facilitates aggregation and impaired clearance from the brain resulting in characteristic NFTs [see review in Ref. (148)]. TBI-induced axonal injury is proposed to be the first perturbation of tau resulting in dissociation from the microtubules. A robust and persistent neuroinflammatory response may then be sufficient to promote phosphorylation, aggregation, and subsequent neurodegeneration; key features of AD (149–151). For example, multiple experimental models of TBI enhance tau pathology that temporally co-exists with gliosis (21, 152, 153). In addition, activated microglia near the injury site release pro-inflammatory cytokines and chemokines that exacerbate tau pathologies (153–155). This is consistent with what is observed in many other tauopathies (156–159), including AD (160); reactive microglia correlate with tau lesions. Together, these studies indicate that chronic neuroinflammation could provoke tau pathology thereby worsening neuronal injury and long-term outcome. Controversy remains in this area though [see review in Ref. (161)], and some data suggest that senescent rather than reactive microglia drive tau pathology and neurodegeneration in AD (162–164). While the relationship between neuroinflammation and neurodegeneration remains complex, a breakdown in communication between microglia and neurons likely sets the stage for neuropathology.

Collectively, human studies show that post-injury tau pathology varies in response to severity, type, and number of brain injuries as well as the time point of post-injury analysis. For example, temporal excision soon after severe TBI reveals axonal and white matter tau phosphorylation in the absence of somatodendritic accumulation (31). Severe TBI resulting in death induces sporadic phosphorylated tau and tau-positive glia but no difference in NFT pathology compared to age-matched controls (165, 166). Together, these studies demonstrate that single TBI induces acute tau phosphorylation but not aggregation. Other studies show that a history of single-incident TBI increases amyloid and tau pathology, neuroinflammation, and white matter degeneration compared to age-matched controls many years after the initial injury (11, 167). Tau pathology, in particular, extended beyond the entorhinal cortex and hippocampus to the cingulate gyrus, superior frontal gyrus, and insular cortex, which was not observed in controls (167). The co-localization of tau pathology and neuroinflammation was not depicted in these studies. Tau pathology has been consistently reported after mild repetitive TBI resulting in CTE. Historic studies on CTE were in boxers, but recent evidence indicates that athletes in many impact-related sports have increased tau pathology followed repetitive mild TBI [see review in Ref. (168)]. Finally, TBI resulting from exposure to an explosive blast causes axonal injury, tau phosphorylation, persistent neuroinflammation, and neurodegeneration characteristic of CTE suggesting that common pathogenic mechanisms mediate outcome in military veterans and repetitively injured athletes (152, 169). These data indicate that tau phosphorylation is a conserved response to TBI regardless of primary insult, but progressive tau pathology occurs in response to repetitive or blast TBI.

Experimental studies indicate that post-injury tau pathology is variable and dependent on multiple factors in non-transgenic rodents. Overall, tau phosphorylation is commonly reported soon after single-incident CCI, weight-drop, FPI, and blast TBI (<7 DPI) (170–174); however, chronic worsening of tau pathology is rare. For example, single blast TBI induces tau phosphorylation in the cortex and hippocampus at 30 DPI (175), with separate groups also reporting persistent hippocampal pathology at 3 months post-injury (176). Also, cortical and hippocampal tau phosphorylation is reported 6 months after moderate CCI but not 6 or 12 months after mild CCI (58, 177). Tau phosphorylation is only part of a potentially pathological process. Following hyperphosphorylation, tau self-assembles, aggregates, and forms NFTs; however, tau oligomers may represent the most toxic and pathologically relevant aggregate. Indeed, oligomeric tau contributes to neurotoxicity by disrupting mitochondrial and synaptic function and strongly correlates with behavioral impairment (178). Recent studies show that fluid percussion TBI induces oligomeric tau in the cortex and hippocampus within 24 h post-injury where it remains elevated compared to shams 2 weeks post-injury (179). Post-injury oligomeric tau was isolated from TBI mice in follow-up studies and injected in the hippocampus of mice overexpressing human tau (hTau). Tau oligomers derived from brain injured mice subsequently caused cognitive dysfunction and the appearance of tau oligomers in hTau mice supporting the notion that tau oligomers are neurotoxic and contribute to tau spreading throughout the brain (180). Accumulating evidence indicates that neuron-to-neuron propagation of tau is a key feature of neurodegenerative tauopathies including AD [see review in Ref. (181)]. Together, these studies implicate soluble tau aggregates as mediators of pathological spreading throughout the brain termed "cistauosis." Thus, abnormal processing of tau is not necessarily the primary mechanism of disease pathogenesis. Recent studies support this concept and indicate that both blast and impact TBI induce *cis* p-tau leading to axonal disruption, tau spreading, and neurodegeneration. Treatment with *cis* p-tau antibody consequently blocked pathological tau spreading and improved functional recovery (182). Collectively, these results indicate that tau alone possesses neurotoxic properties that mediate recovery following TBI.

Few experimental TBI studies have been performed in tau transgenic mice without concurrent amyloid pathology. Single-incident mild CHI TBI in aged hTau transgenic mice that express all six isoforms of hTau in absence of murine tau did not worsen

tau phosphorylation or induce tau aggregation 3 weeks post-injury (153). A separate group of investigators examined the acute and chronic effects of moderate lateral FPI in a similar hTau mouse model, mouse tau knockout expressing wild-type human transgene, and found that the macrophage response to TBI was enhanced compared to control TBI and sham mice at 3 DPI with no influence on tau phosphorylation (183). This was confirmed with immunohistochemistry examining expression of CD45, F4/80, and CD68. By 120 DPI hTau TBI mice displayed increased tau pathology in the cortex and hippocampus and a persistent macrophage response that correlated with deficits in spatial search strategies to complete a memory task (183). Incorporation of flow cytometric techniques facilitated identification of four distinct macrophage populations at 120 DPI: (1) $CD11b^{low}/CD45^{low}$ microglia, (2) $CD11b^{high}/CD45^{low}$ microglia, (3) $CD11b^{+}/CD45^{int}$ microglia, and (4) $Ly6C^{+}/CD11b^{+}/CD45^{high}$ macrophages. The $CD11b^{low}$ microglia expressed the lowest levels of CD45, followed by the $CD11b^{high}$ and the $CD45^{int}$ groups characteristic of reactive microglia, while the peripheral macrophages were the highest expressers of CD45. A significant proportional reduction was identified in hTau TBI compared to wild-type TBI mice in all three microglial sub-populations at 120 DPI. No significant differences were observed in the proportion of $CD11b^{+}/CD45^{high}$ cells between brain- and sham-injured hTau and wild-type mice. $Ly6C^{low}$ and $Ly6C^{high}$ microglia were significantly reduced in the hTau TBI mice, but $Ly6C^{low}$ macrophages persisted at significantly higher numbers compared to the hTau sham-injured group. The authors speculate that $Ly6C^{low}/CD11b^{+}/CD45^{high}$ cells represent $CX3CR1^{+}$ patrolling macrophages (184), and that $Ly6C^{+}/CD11b^{+}/CD45^{int}$ microglia represent inflammatory $CCR2^{+}$ monocyte-derived macrophages, differentiating in the CNS tissue environment. Without detailed cell-specific analysis of cytokine and chemokine expression, the true nature of these cell populations remains unclear. For the first time, these results show that a single TBI significantly changes the proportion of reactive microglia and macrophages within the brains of hTau mice compared to wild-type mice many months after TBI (183). These data indicate that the presence of wild-type hTau is sufficient to alter the macrophage response to single-incident TBI.

Collectively, these studies confirm the vulnerability of the brain to tau pathology following single-incident TBI. Indeed, both clinical and experimental studies consistently report tau phosphorylation soon after TBI; however, the presence or absence of tau phosphorylation alone is not sufficient to define tau pathology and may represent a transient effect of TBI. Furthermore, the role of tau oligomers is very limited in the context of TBI and represents an important avenue of study for future experiments. The prion-like properties associated with abnormal tau implicate the protein itself as an initiator of disease pathogenesis. As a result, the relationship between damaged neurons and other cells types remains unclear and the question remains, which cell is driving post-injury pathology? While recent experimental studies demonstrate a unique macrophage response to TBI that correlates with tau pathology and behavioral impairment, one is left wondering whether or not the abnormal tau caused the altered inflammatory response or the altered inflammatory response caused the abnormal tau? Certainly, use of tau knockout mice or CCR2 and CX3CR1 knock-in/knock-out mice could provide insight into these questions. In addition, the time course of pathology must be a priority. Defining age-related pathology requires aging as a primary variable of interest and the temporal course of disease pathology should not be undervalued.

TBI AND COMBINED EFFECT OF AMYLOID AND TAU-RELATED PATHOLOGY

The combined effect of amyloid and tau pathology has gained recent attention over the last 10 years; however, results from non-transgenic rodent studies remain variable. The presence of Aβ and tau pathology appears to be dependent on the injury model used and the post-injury time point. Both fluid percussion and moderate CCI TBI induce Aβ and tau pathology at acute (3 and 7 DPI) and chronic (6 months post-injury) time points in rats (177, 185, 186), but other groups report no difference in Aβ or tau levels at 2 and 4 weeks post-injury (187). PBBI decreased full length APP at 3 and 7 DPI but increased beta-secretase C-terminal fragments of APP. Both Aβ40 and Aβ42 were increased at 7 DPI, but the authors explain that detection was difficult due to low expression. Similarly, full length tau decreased at 3 and 7 DPI but oligomeric tau was elevated at 4 h and 7 DPI (188). Out of these studies, only one reported that increased Aβ and tau pathology occurred in conjunction with neuronal loss and increased MHC-II immunoreactivity several months post-injury (177).

Given that Aβ and abnormal tau are hallmark features of AD, transgenic mice harboring mutations in both APP and MAPT are more often used to characterize the relationship between TBI and AD. Use of these models provides investigators with an opportunity to study the interaction of Aβ and tau pathology following TBI, but the clinical relevance of these models often comes into question. To date, no mutation in MAPT is causative in development of AD thereby restricting the translation of results. Nonetheless, accumulation of Aβ and tau pathology occurs as a result of normal aging [see review in Ref. (189)] and, therefore, the relevance of these abnormal proteins as mediators of response to and recovery from TBI remains applicable.

Variations of CCI have been used to examine the effects of TBI mouse models of AD with amyloid and tau mutations *via* overexpression of transgenes. For example, a series of studies examining moderate CCI in 3xTg-AD mice revealed a temporally and anatomically distinct increase in intra-axonal Aβ and tau phosphorylation between 24 h and 7 DPI (21, 25). Follow-up studies revealed that post-injury Aβ and tau pathology could be improved *via* inhibition of γ-secretase or c-Jun N-terminal kinase (JNK), respectively (21, 190). Interestingly, a recent report shows that post-injury JNK inhibition improves amyloid and tau pathology, neuroinflammation, BBB disruption, synaptic loss, and neurodegeneration in non-transgenic mice 7 DPI (191). Thus, the JNK pathway may be a relevant therapeutic target influencing multiple pathological processes.

In addition, the effect of *ApoE4* allele was examined in the 3xTg mice after TBI. *ApoE4* is a primary genetic risk factor for late-onset AD and has been associated with worsened outcome

after TBI (192–196). While 3xTg-ApoE4 mice displayed increased post-injury APP accumulation compared to 3xTg mice with the *ApoE2* or *ApoE3* allele, TBI did not influence intra-axonal Aβ40 and Aβ42 or tau pathology 24 h post-injury. These results demonstrate that axonal injury may be a primary effect of *ApoE4* genotype following TBI but the interaction effect of *ApoE* and tau pathology remains unclear in this experimental model of TBI (197). Collectively, these data indicate that genetic predisposition to AD drives independent mechanisms that promote post-injury amyloid and tau pathology.

Other studies have examined tau pathology in mouse models of AD with genetic predisposition to developing only amyloid pathology *via* inclusion of mutant human transgenes. A recent study examining CCI in APP/PS1 mice revealed chronic region-specific changes in Aβ with no change in tau pathology 16 weeks post-injury (198). For example, Aβ plaques decreased in the perilesional area after TBI which correlated with increased expression of genes involved in Aβ clearance (198). Finally, CCI in Tg2576 mice, which overexpress mutant APP, increased Aβ, tau phosphorylation, and inflammatory cytokines IL-1β and TNF-α 3 DPI. Inhibition of GSK *via* treatment with the flavonoid luteolin attenuated this response (199), but the long-term and functional consequences of this intervention remain unknown.

Together, these studies indicate that choice of experimental TBI model and rodent model (rat or mouse, transgenic or non-transgenic) influence the temporal appearance of post-injury amyloid and tau pathology through independent mechanisms. Alternatively, a common mechanism may mediate post-injury amyloid and tau pathology in a temporally distinct manner which may vary between transgenic and non-transgenic rodent models. Finally, one could hypothesize that accumulating pathological proteins in transgenic rodents substantially mediates the brain's ability to respond to injury by priming the inflammatory environment *before* TBI. Cell-specific inflammatory profiles of microglia and monocytes are not routinely performed in transgenic mice prior to TBI, therefore this effect remains unknown. In summary, the combined effect of amyloid and tau pathology following TBI is complex and likely dependent on multiple factors that are time- and injury severity-dependent. We propose that future studies look beyond accumulation of amyloid and tau as primary dependent variables of interest and consider interaction effects of inflammation, amyloid, and/or tau as mediating factors of post-injury outcome measures. Increasing evidence, as described in the following sections, clearly shows that pre- and post-injury immune stressors that elicit macrophage reactivity influence response to and recovery from TBI.

PRE-INJURY PERIPHERAL IMMUNE CHALLENGE IMPROVES RECOVERY FOLLOWING TBI

Neuroprotective preconditioning occurs when a moderate primary stimulus protects the CNS from a secondary stimulus. The goal is to use a sub-threshold inflammatory stimulus to precondition a neuroprotective response to a secondary stimulus. For example, peripheral LPS treatment is neuroprotective against stroke, ischemia, and higher-dose LPS treatments (200). Similar effects have been reported in experimental models of TBI. For example, a single i.p. dose of LPS (0.1 mg/kg) 5 days before CCI reduced CD68 and increased IL-6 expression in TBI mice, which correlated with decreased contusion volume and improved behavioral recovery (201). Follow-up studies revealed that a single i.p. dose of LPS (0.1 and 0.5 mg/kg) 5 days before CCI delayed post-injury kindling epileptogenesis. In addition, pre-injury LPS treatment attenuated neuronal loss, IL-1β, and TNFα overexpression in the hippocampus (202). More recently, pre-injury treatment with LPS (1.0 mg/kg, single i.p. dose for 4 days) reduced neuronal death and lesion volume after lateral cryogenic brain injury (61). The authors conclude that microglial reactivity induced by 4 peripheral pre-injury LPS injections offers post-injury neuronal protection. Indeed, Chen and colleagues demonstrate that peripheral LPS treatment increases cortical expression of M2-related genes, such as *Ym1*, *Socs3*, *Il4ra*, *Ptprc*, *Cd163*, *Il1ra*, *Mrc1*, and *Arg1* (61). While the appearance of AD-related pathology was not examined in any of these studies, the data support a neuroprotective role of reactive microglia and indicate that pre-injury immune challenge significantly alters response to and recovery from brain injury, in part, *via* modulation of macrophage reactivity and cytokine production.

POST-INJURY PERIPHERAL IMMUNE CHALLENGE WORSENS RECOVERY FOLLOWING TBI

Increasing evidence shows that TBI induces a persistent pro-inflammatory profile in microglia, but the functional consequence of this dysfunction is still under investigation. For example, single CCI in adult B6 mice induced chronic microglial reactivity 12 months post-injury. Highly reactive microglia were detected near the lesion cavity and characterized by increased expression of MHC-II, CD68, and NADPH oxidase (56). Midline FPI also induced features of primed pro-inflammatory microglia up to 1 week post-injury, which included elongated, rode-shaped Iba1$^+$ cells that were also MHC-II and CD68 positive (203). Follow-up studies revealed that MHC-II mRNA and protein expression increased specifically in microglia after FPI and correlated with Iba1 reactivity and amoeboid morphology at 30 DPI (204). These data align with human studies showing a persistent post-injury microglial inflammatory profile. For example, inflammatory cytokines IL-6 and TNFα are detected in the CSF up to 12 months post-severe TBI and correlate with functional impairment and disinhibition (205, 206). Immunohistochemical analysis of autopsied brains revealed increased CD68$^+$ and CR3/43$^+$ (MHC-II$^+$) reactive microglia several months post-injury. The presence of TAI increased the immunoload of microglial reactivity, particularly in the white matter (207). Separate studies demonstrate that CR3/43$^+$ cells associate with increased APP accumulation 2 weeks post-injury and myelin basic protein 2–8 years post-injury (11). Together these data confirm that TBI induces persistent macrophage inflammation; however, the cell-specific role of microglia and monocytes remains unknown. Without cell-specific analysis of microglia and monocytes, the distinct

role of each cell type is unclear and, therefore, not targetable with therapeutics. For example, macrophage reactivity could be the result of some other pathological process or macrophage reactivity could be perpetuating post-injury pathology. Additional work is needed to address these outstanding concerns.

Recent data demonstrate that TBI induces a primed microglial phenotype, defined by altered morphology and increased expression of MHC-II and CD68 (204, 208). Primed microglia do not display acute reactivity but instead become hyper-reactive after immune stimulation [see review in Ref. (66)]. This effect has been observed in experimental models of aging. For example, aged rodents display microglial priming via increased expression of MHC-II, complement receptor 3 (CD11b) and altered morphology (209, 210). Aged animals challenged with lipopolysaccharide (LPS) display increased microglial expression of IL-1β compared to adult mice, which results in prolonged sickness behavior (211) and a depressive-like phenotype (212). Follow-up studies revealed that LPS challenge in aged mice also promoted increased hippocampal expression of IL-1β, IL-6, and TNFα as well as spatial memory impairments (213, 214). Moreover, a similar effect is induced by midline FPI in adult mice. Midline FPI induces acute microglial activation, recruitment of peripheral cells, and motor impairment; however, many of these effects are transient. Only glial reactivity persists 30 DPI with deramified microglia maintaining increased expression of MHC-II. Peripheral LPS challenge at 30 DPI caused an exaggerated microglial response in TBI mice characterized by increased MHC-II, IL-1β, and TNFα expression and depressive-like behaviors compared to TBI mice given saline (204). Subsequent studies confirmed that LPS at 30 DPI exaggerated memory recall deficits in TBI mice as well (208). Another group has since reported that LPS 5 DPI causes an exaggerated inflammatory response in TBI rats (*via* impact acceleration) which is associated with depressive-like behavior and cognitive impairments 3 months post-injury (215). These results emphasize the chronic nature of the inflammatory response to TBI and confirm that subsequent post-injury immune challenges influence outcome and elicit exaggerated behavioral deficits.

Together, these studies indicate that primed microglia potentiate brain pathology and behavioral decline in aging and after CNS injury. While there are many similarities between age- and injury-related microglial priming, the cumulative effect of aging and TBI in microglial priming remains unknown and may be critical in determining the relationship between TBI and development of age-related neurodegenerative disease such as AD. For example, recent analysis of human brain samples revealed that LPS and *E. coli* K99 proteins were increased in AD brains compared to controls. LPS co-localized with Aβ40 and Aβ42 around amyloid plaques and near blood vessels (216). Multiple experimental studies show that peripheral LPS induced neuroinflammation, accumulation of Aβ, tau pathology, and cognitive impairment in non-transgenic rodents (217–219) although variable Aβ and tau pathology is apparent in transgenic mouse models of AD after peripheral LPS treatment (220–224). Moreover, peripheral LPS treatment after ischemia-hypoxia induced Aβ that co-localized with myelin aggregates in rats (225). While the mechanism(s) by which LPS enters the brain in unknown, these studies lend support to the notion that infection may be associated with development of AD. Thus, post-TBI infection that stimulates an inflammatory response may have a significant effect in long-term recovery.

Repetitive TBI as a Post-Injury Immune Challenge

Mounting evidence indicates that neuroinflammation and microglial priming is a factor in repetitive TBI as well. In this case, the first TBI is the priming event and subsequent brain injuries cause an exaggerated inflammatory response that promotes pathology. Repetitive TBI through participation in contact sports is associated with chronic cognitive impairment, including development of CTE, a neurodegenerative disease characterized by abnormal tau accumulation in the sulci of the cortex (226). While Aβ plaques are present in some cases of CTE, the distribution and location is distinct from that occurring in AD (227–229). Glial reactivity is a common feature of CTE and includes astrocytic accumulation of abnormal tau and microglial reactivity (152, 226). For example, recent PET imaging studies reveal increased TSPO binding in retired NFL players in the hippocampus, entorhinal cortex, parahippocampal cortex, and supramarginal gyrus compared to age- and sex-matched controls without a history of repeated brain injury (230). This supports other studies showing increased $CD68^+$ microglia in the brains of American football players, which partially mediated coincident tau pathology (231). Together, these data indicate that chronic neuroinflammation mediates AD-related pathology following repetitive TBI and the inter-injury time interval may be critical in this response.

One is faced with many challenges when trying to summarize data from experimental studies describing the relationship between repetitive TBI, neuroinflammation, and AD. First, a universal experimental model of repetitive TBI is not established. Thus, there is a great deal of variability in the number of injuries and timing between injuries in published reports. Second, the role of amyloid is underexplored in experimental models of repetitive TBI, which is predominantly characterized by tau pathology. Third, control animals after each TBI are not always included. For example, comparisons are often made between sham mice and brain injured animals that received the highest number of repetitive brain injuries. As a result, data describing inflammatory changes between injuries remain limited. Finally, most studies use Iba1 or morphological analysis to define reactive macrophages at a sub-acute time point after the final brain injury. Repetitive TBI consistently alters astrogliosis and microgliosis up to 1 year post-injury with a positive correlation between number of injuries and gliosis (232). While these data are important, they provide a restricted view of the cell-specific role of microglia and monocytes and do not define the cell-specific inflammatory state between injuries. To date, no studies could be found that define inter-injury macrophage changes in experimental models of repetitive TBI. Thus, the following paragraphs will briefly describe the incidence of Aβ and tau pathology as well as macrophage reactivity at post-injury time points following repetitive TBI.

Animal models of repetitive mild TBI are described in a recent review, which includes references to development of post-injury

Aβ and tau pathology (232). While severity of repetitive TBI is typically referred to as "mild," the number of injuries varies from 2 to 10 across several days or weeks. A recent highly repetitive mouse model of TBI including 30 injuries has also been characterized (59). Typically 1 or 2 brain injuries are administered per injury day. Non-transgenic mice exposed to repetitive mild TBI consistently show increased APP, phosphorylated tau, and behavioral impairment at chronic post-injury time points (232). Only two reports examining repetitive TBI in Tg2576 mice showed increased Aβ in addition to behavioral impairment at chronic post-injury time points (165, 233). Similarly, only transgenic tau mouse models [T44 (one mouse), hTau] displayed NFT pathology following repetitive TBI (153, 234). Macrophage reactivity was only reported in 1 of these 4 studies, and indicated that repetitive TBI in aged hTau mice resulted in increased CD45 immunoreactivity in the cortex, corpus callosum, and hippocampus 3 weeks post-injury (153). Together, these data are similar to single TBI experimental studies and show that TBI alone is not sufficient to induce amyloid or tau aggregation in non-transgenic rodents. The presence of pathological proteins (e.g., amyloid and tau oligomers or fibrils) in transgenic rodents at the time of TBI is sufficient to promote aggregation. Nonetheless, a recent report indicates that 30 mild TBI's do not alter Aβ and tau pathology in 18-month-old 3xTg mice (59). In summary, these data confirm the complexity of repetitive TBI and strongly emphasize the need for more research in this area. Furthermore, a uniform experimental model is required to confirm the inter-relationship between repetitive TBI, neuroinflammation, and AD-like pathology.

What about peripheral immune challenge after repetitive TBI? One group considered this question and determined that timing of LPS treatment mediated a beneficial or detrimental post-injury effect in rats. For example, LPS treatment 1 day after repetitive mild TBI (3 TBI's, 5 days apart) increased macrophage reactivity but decreased production of inflammatory cytokines and reduced neuronal injury (235). Delayed LPS treatment 5 days after repetitive TBI increased inflammatory cytokines, worsened neuronal damage including phosphorylation and aggregation of tau, and impaired behavioral recovery (235). These results highlight the temporal immune response to TBI and indicate that delayed post-injury immune challenges are detrimental to outcome.

MACROPHAGE ELIMINATION ALTERS RECOVERY FOLLOWING TBI

If the macrophage response is critical in mediating outcome following TBI, removal of microglia and/or monocytes should substantially alter recovery. Studies with CCR2 and CX3CR1 knock-in/knock-out mice demonstrate that permanent interruption of the microglial or macrophage response to TBI does not offer optimal protection after injury. Alternatively, various pharmacologic agents are available to transiently interrupt the microglial and macrophage response to injury. While the use of these agents is limited in experimental TBI studies, several groups have reported that microglial elimination [*via* colony-stimulating factor 1 receptor (CSF1R) inhibition] improves behavioral performance and synaptic functioning independent of Aβ accumulation in multiple transgenic mouse models of AD (3xTg, 5xFAD, APP/PS1) (236–238). Similarly, CSF1R inhibition after a chronic hippocampal lesion model of brain injury improved behavioral recovery, reduced pro-inflammatory molecules, and increased dendritic spines (239). Recent studies using the same injury model confirmed that post-injury microglial depletion followed by microglial repopulation improves behavioral recovery, attenuates the lesion-induced neuroinflammatory response, and increases dendritic spin densities despite extensive neuronal loss in the hippocampus (240). Another group examined the role of microglia in axonal damage following repetitive TBI by using CD11b-TK (thymidine kinase) mice, which require valganciclovir to deplete macrophages. In these experiments, low and moderate doses of valganciclovir reduced CD11b cell populations with no effect on axonal injury, silver staining, or APP accumulation at sub-acute post-injury time points (241). Discrepancies between the brain injuries studies are likely due to multiple factors, including (1) injury model; (2) post-injury time points; (3) post-injury

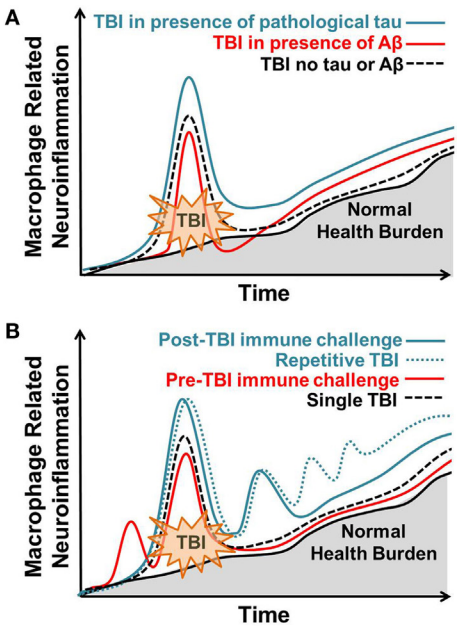

FIGURE 1 | Macrophage-related response to brain injury varies in response to previous, coincident, and subsequent immune stressors. Normal, age-related health burden is depicted with a solid black line and gray shading. **(A)** Traumatic brain injury (TBI) in the presence of pathological tau (solid blue line) results in an enhanced macrophage response to TBI that remains elevated at chronic post-injury time points. TBI in the presence of Aβ (solid red line) results in an acute blunted macrophage response that increases at chronic post-injury time points. TBI occurring in the absence of tau or Aβ (dotted black line) results in acute macrophage-related neuroinflammation that subsides over time. **(B)** Post-injury peripheral immune challenge (solid blue line) causes a hyper-active macrophage response correlating with behavioral dysfunction. Repetitive post-injury immune challenge (dotted blue line), similar to what is observed in repetitive TBI, increases macrophage-related neuroinflammation and correlates with the advanced neuropathology. Pre-injury peripheral immune challenge at sub-threshold levels (red line) attenuates the post-injury macrophage-related inflammatory response to TBI. Single TBI (dotted black line) results in acute macrophage-related neuroinflammation that subsides over time. Over time, macrophage-related neuroinflammation increases with normal health burden.

outcome measures (only one considered the effect on APP and tau pathology); (4) CSF1R targets microglia specifically while CD11b$^+$ cells include microglia and macrophages; and (5) CSF1R eliminated >90% of microglia while low and intermediate dose of valganciclovir depleted 35 and 56% of CD11b$^+$ cells, respectively. Nonetheless, these studies highlight the potential therapeutic relevance of targeting microglia and macrophages to modulate post-injury outcome and further indicate that neuroinflammation is a critical mediator of post-injury pathology.

CONCLUSION

Experimental models are a valuable resource in identifying the underlying biological pathways that link TBI to AD. Both TBI and AD are complex neurodegenerative pathologies that elicit a central and peripheral immune response. This review showcases the dynamic nature of post-injury macrophage-mediated inflammation in promoting post-injury Aβ and tau pathology. Throughout the review, several themes emerged that are notable and depicted in **Figure 1**. First, the inflammatory response to TBI is not absolute and is influenced by previous and subsequent inflammatory challenges. This is best reflected in studies with LPS administration before or after TBI. Importantly, in both instances LPS administration altered outcome from TBI. Certainly, there is a specific cascade of inflammatory events that occur after TBI, but these studies indicate that subtle alterations in this response are possible and can influence outcome. Second, accumulation of Aβ and tau phosphorylation are routinely considered primary dependent variables in experimental studies, but these pathological features do not often correlate with neuronal loss or behavioral impairment. Furthermore, many studies report that TBI does not influence Aβ and/or tau pathology leaving one to question the true role of these proteins in post-injury outcome. Both Aβ and tau phosphorylation are reported in normal aging and could, therefore, influence the brain's response to TBI without causing AD. Thus, accumulation of Aβ and tau phosphorylation could be viewed as part of the injury process instead of a result of the injury. Third, additional factors must account for the resistance of rodents to develop Aβ and tau pathology after TBI. This could be due, in part, to intrinsic differences between human and rodent APP and tau. Given that multiple mouse models of AD display an altered inflammatory response to TBI, it is possible that accumulation of pathological proteins alters the neuroinflammatory environment in a way influences the brain's response to injury. One could speculate and suggest that low-molecular weight pathological proteins "prime" the brain to respond to TBI. The role of Aβ and tau in this "priming" is potentially distinct and may include beneficial and detrimental consequences depending on age at injury and time of post-injury analysis. Finally, the distinct role of microglia and monocytes in TBI requires additional investigation and characterization. Targeting these cell types independently may provide new avenues for therapeutic intervention. Accumulating evidence shows that transient interruption of the macrophage response to TBI could improve outcome. Moving forward, we must appreciate the continuous nature of inflammation and consider previous, consequent, and subsequent immune challenges as mediators of post-injury outcome.

AUTHOR CONTRIBUTIONS

OK-C wrote the manuscript; JG provided expertise in the neuroinflammation and edited the manuscript.

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REVIEW
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Microglia and Beyond: Innate Immune Cells As Regulators of Brain Development and Behavioral Function

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Innate immune cells play a well-documented role in the etiology and disease course of many brain-based conditions, including multiple sclerosis, Alzheimer's disease, traumatic brain and spinal cord injury, and brain cancers. In contrast, it is only recently becoming clear that innate immune cells, primarily brain resident macrophages called microglia, are also key regulators of brain development. This review summarizes the current state of knowledge regarding microglia in brain development, with particular emphasis on how microglia during development are distinct from microglia later in life. We also summarize the effects of early life perturbations on microglia function in the developing brain, the role that biological sex plays in microglia function, and the potential role that microglia may play in developmental brain disorders. Finally, given how new the field of developmental neuroimmunology is, we highlight what has yet to be learned about how innate immune cells shape the development of brain and behavior.

Keywords: microglia, brain development, sex differences, synaptic pruning, neurodevelopmental disorders, early life stress, inflammation, behavior

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PURPOSE OF THIS REVIEW

Microglia, the brain's primary resident immune cells, were named and first studied by Pio del Rio Hortega in the 1920s. Since that time, the role of immune cells in the brain and behavior following injury, illness, or infection has been well appreciated. Innate immune cells clearly play a role in the etiology and disease course of multiple sclerosis, Alzheimer's disease, traumatic brain and spinal cord injury, and brain cancers. Thus, the potential is high that modulating neuroimmune signaling and function will be a viable target for therapeutic interventions. However, innate immune cells in the brain do more than respond to injury and pathological conditions. The last decade has seen an exponential growth in interest in immune cells as regulators of normal and abnormal brain development in response to early life perturbations. In this review, we pursue two main goals. First, we summarize what is currently known about microglia during normal brain development as well as in response to early life stress, infection, and other early life exposures. We focus particularly on areas in which developmental microglia function may be misunderstood, given the much larger and sometimes contrasting knowledge-base on microglia function in the context of injury or neurodegeneration. Second, in each section of the review, we highlight areas of future interest, including where more is unknown than known, where this young field is headed, and where the field may need to refine and

complicate the traditional, canonical and potentially dogmatic views of how microglia shape brain function.

OVERVIEW OF MICROGLIAL DEVELOPMENT

Microglia are the primary innate immune cells of the brain. They colonize the brain early in brain development. The mechanisms driving microglia colonization and differentiation have only recently been described. In humans, primitive microglia/macrophages are seen near the mesenchymal tissue capillaries before their appearance in neural tissue around 4.5 weeks of gestation and are present in the neural tissue by 5.5 weeks of gestation (1, 2). In rodents, a subset of $CD45^{-}c\text{-}kit^{+}$ erythromyeloid precursors from the yolk sac use blood circulation to travel to and colonize the mesenchyme surrounding the neural tube beginning at embryonic day (E) 8 (3, 4). In the mesenchyme, the progenitor microglia begin expressing fractalkine receptor (e.g., CX_3CR1) and downregulate c-kit starting around E9.5 (4, 5). The CX_3CR1 + microglia progenitors invade the neuroectoderm likely using matrix metalloproteinases, at which point the contribution of peripheral progenitors slows or stops (4, 5). Microglia precursor formation initially depends on cell survival factor (CSF) 1-receptor (CSF1-R) signaling and the transcription factors, PU.1 (SPl1) and interferon regulatory factor 8 (IRF8) (4–6). Interleukin (IL)-34 is likely the predominant CSF1-R ligand during development, as CSF-1 knockout mice do not lack microglia and IL-34 is expressed at greater levels during early brain development (7–9). Microglia colonization can be influenced by fibronectin, macrophage migration inhibitory factor (MIF), fractalkine (e.g., CX3CL1), and CXCL12 (10–13). Once colonization has occurred, microglia locally proliferate in the brain until the second week of postnatal life in rodents. After this peak in numbers, microglia number decreases to adult levels (14–16). This developmental trajectory is summarized in **Figure 1**.

Microglia are phenotypically and developmentally distinct from peripheral macrophages and other tissue-resident macrophage populations, such as Kupffer cells and aveolar macrophages (14, 17–21). Microglia arise from yolk-sac fetal macrophages, whereas other tissue macrophages arise from precursors generated slightly later in development (20, 21). Hematopoietic cells do not contribute to microglia homeostasis during normal development and adulthood (5). However, peripheral hematopoietic cells may contribute to the microglia pool in the brain in pathological circumstances. For example,

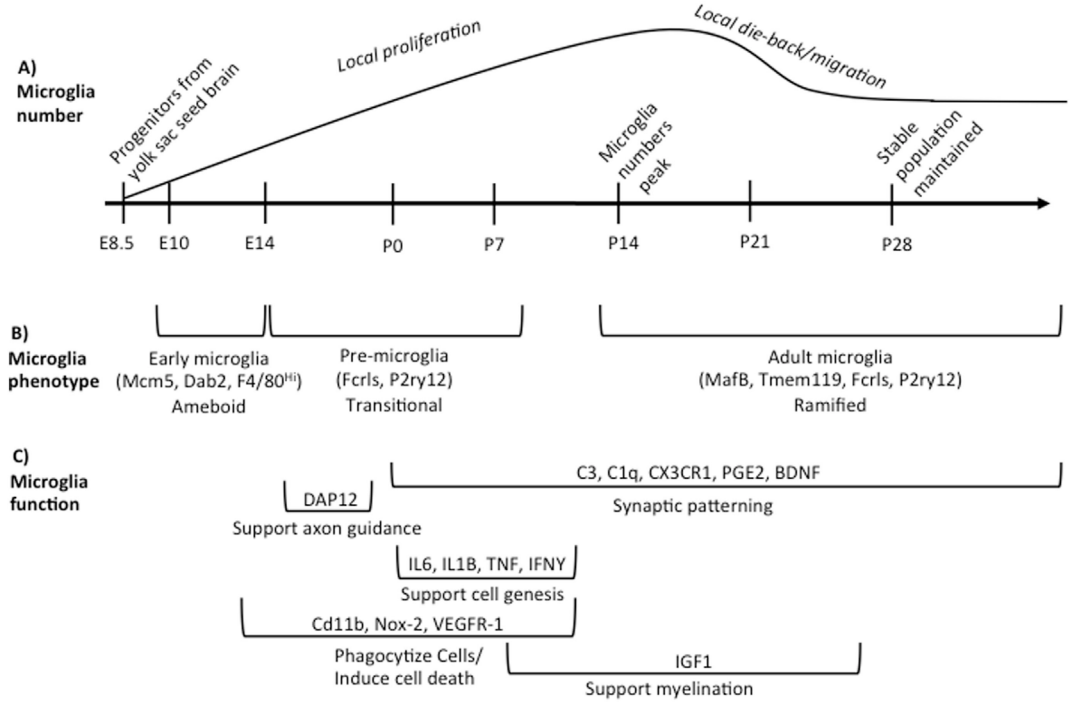

FIGURE 1 | Overview of microglia during brain development. **(A)** illustrates the developmental time points when microglia colonize and proliferate in the developing brain, beginning on embryonic day (E) 8.5. Microglia numbers peak in the rodent brain at postnatal day (P) 14 following local proliferation. Microglia then either die back or migrate from region to region until adult numbers are reached by P28. **(B)** indicates the different phenotypes of microglia across development based on unique gene expression patterns, listed in parentheses, and somewhat distinct morphologies. The brackets refer back to the developmental timeline depicted above in **(A)**. **(C)** summarizes our current state of knowledge regarding which processes of development microglia have been shown to regulate, as well as the molecular factors involved. The brackets refer back to the developmental timeline depicted above in **(A)**, illustrating when research to date has shown that microglia are involved in a given process. These data do not preclude the (likely) possibility that microglia regulate each developmental process beyond the time window indicated, but represent our current state of knowledge. **Table 1** lists the publications that were used to design **(C)**.

following chronic stress and irradiation of the brain that compromise the blood brain barrier, peripheral hematopoietic cells can invade the neural tissue and become part of the microglia/macrophage pool in the parenchyma (22, 23). Together, these data suggest that microglia have a unique developmental origin and tissue environment that drives their specialized development.

DEVELOPMENTAL MICROGLIA ARE DISTINCT

Microglia have unique gene expression profiles during different phases of development. Matcovitch-Natan et al. (24) found that microglia express distinct sets of genes that can divide microglia into three distinct groups: early (E10.4-14), pre-microglia [E14-postnatal day (P) 9], and adult microglia (P28 and on). Bennett et al. (14) also found similar developmental changes in microglial gene expression. Some canonical microglia genes are expressed very early in microglia development (e.g., Fcrls, P2ry12) whereas others are only expressed in adult microglia (e.g., MafB, Tmem119) (14, 24). Microglia in the prenatal and early postnatal brain have distinctly different morphologies than microglia in the adult brain. They are largely non-ramified, instead possessing an ameboid morphology until the early postnatal period (25). Bennett et al. (14) also found that microglia begin to adopt a mature phenotype around the end of second postnatal week of life similar to Matcovitch-Natan et al. (24) as indicated by the expression of Tmem119 and a predominately ramified morphology. These developmental differences in microglia gene expression are summarized in **Figure 1**.

The ameboid morphology seen in developing microglia led to the natural conclusion that microglia were in a constitutively "activated" state in the developing brain, possibly behaving very differently than adult microglia. Recent research has shown that developing microglia do behave differently than adult microglia, but are not "activated" in the same way adult microglia are in response to inflammation or neurodegeneration. For example, there is not substantial gene expression overlap between lipopolysaccharide (LPS)-stimulated microglia from the adult brain and microglia from a control neonatal brain (14). Additionally, a unique Cd11c + microglia population exists in the developing white matter areas; yet, these Cd11c + microglia are not similar to Cd11c + microglia present in a rodent model of multiple sclerosis (experimental autoimmune encephalomyelitis) in terms of their gene expression profile (26). In contrast, microglia isolated from different neurodegenerative disorders share a gene expression profile (high Trem2, ApoE, and Cd11c) (27), which underscores that developmental microglia are phenotypically distinct from "activated" microglia seen in neuropathological conditions. Microglia do express some markers during development that are commonly associated with "activation" or neurodegeneration such as CD11c and CD68, but together the aforementioned data show that developmental microglia are not interchangeable with adult "activated" microglia. Indeed, notions of "activated" or "quiescent" microglia are certainly too simplistic for the dynamic and busy cells during the developmental period. Instead, the field has quickly moved beyond such nomenclature in favor of assessing microglia gene expression, phagocytic capacity, altered density, and/or ultrastructure [for example, see Ref. (24, 28–30), respectively]. Developmental microglia are clearly unique and easily distinguishable from adult "activated" microglia, thus analysis of microglia by morphology alone is likely to be uninformative, or at its best, an imperfect proxy measure of their gene expression profile or function. Other recent studies also suggest that macro-environmental challenges induce complex changes in microglial gene expression during development that differ from those changes seen later in life in response to perturbations (see The Macro-Environment Drives Microglia Function), again underscoring that developing microglia are unique.

DIVERSE MICROGLIA FUNCTIONS IN DEVELOPMENT

Microglia regulate brain development primarily through two routes: the release of diffusible factors and phagocytosis. Microglia phagocytize many products in the brain, including synaptic elements, living cells, dying or dead cells, and axons. Microglia also support myelination/oligodendrogenesis, neurogenesis, axon fasciculation, induce cell death or cell survival, and stimulate synaptic formation and maturation *via* the release of diffusible factors (11, 31–40). Many factors contribute to microglia phagocytosis of cells, such as vascular endothelial growth factor (VEGF), NADPH oxidase (Nox) 2, and Tyrobp/DAP12 either through recognition of cells marked for removal or by inducing cell death (40, 41). Microglia phagocytosis of neural progenitors increases toward the end of the developmental neurogenesis period in rodents and primates; however, it is unknown if this increase is instigated by microglia or if progenitor cells begin to express a tag that recruits microglia (31). There are several "tags" that regulate cellular phagocytosis such as phosphatidyl serine, complement, calreticulin, ATP, and sialic acid (42, 43). Receptors on microglia that bind to these "tags" include MERTK, vitronectin, CR3, siglecs, and SIRP1α. Microglia also continue to shape the rate of cell genesis throughout life in the hippocampus *via* phagocytosis (44).

Microglia also phagocytize synapses, which has been most elegantly demonstrated in the dorsolateral geniculate nucleus of the thalamus (dLGN). During the early postnatal period, microglia phagocytize "weak" retinal ganglion synapses in the dLGN by recognizing complement component 3 (C3) through the complement receptor 3 (CR3) (36, 45, 46). Decreased retinal ganglion activity and/or increased transforming growth factor (TGF) β signaling in retinal ganglion cells stimulates the synthesis of complement component 1q (C1q) by retinal ganglion cells, which potentially initiates the complement cascade (36, 45, 46). The complement system is not the only signaling system that mediates phagocytosis. Recent work has identified interleukin-33 as an astroglial-secreted factor that regulates microglia synaptic phagocytosis through the interleukin 1 receptor-like 1 (IL1RL1) in the developing thalamus and spinal cord (47).

In the dLGN, there are two periods of intense microglial synaptic phagocytosis. The first period is during the early neonatal period after initial synapses are formed, and the second period is during

the juvenile when the fine connections are pruned (48). Microglia also prune synapses in the hippocampus during the second week of life (12). Other factors that regulate synaptic pruning include Class I major histocompatibility complex genes (H2-K^b and H2-D^b) and pentraxins (49, 50); however, it is unknown if microglia are directly involved in these synaptic pruning mechanisms. H2-K^b and H2-D^b colocalize with C1q, suggesting that microglia may be involved (50). Additionally, microglial synaptic pruning has only been closely investigated in the developing dLGN and hippocampus; thus, it is unknown whether or how microglia contribute synaptic pruning in other brain areas.

Microglia support cell genesis and/or cell health through the synthesis and release of insulin-like growth factor 1(IGF1) and a variety of cytokines that include tumor necrosis factor (TNF) α, IL 1β, IL6, and interferon (IFN) γ (26, 32, 37). Microglia can also stimulate dendritic spine and synapse formation *via* the release of brain-derived neurotrophic factor (BDNF), prostaglandin E2 (PGE2), and IL-10 (33, 51, 52). The major mechanisms and molecules through which microglia have been shown thus far to regulate brain development are summarized in **Figure 1** and **Table 1**.

Despite this knowledge, we have only scratched the surface of understanding microglia function during development, and the situation is likely more complicated than it seems. Microglia support cell survival in layer V of the developing neocortex and yet oligodendrocytes do so as well (39, 53). Thus, microglia could be contributing to cell survival indirectly by supporting oligodendrogenesis or through an additive effect on oligodendrocyte-derived growth factors. Microglia also support cell genesis in the developing brain through release of several cytokines (37), and it is unknown whether cytokine receptors are expressed on neural progenitor cells during development. These receptors would be necessary for direct stimulatory effects of cytokines on neurogenesis to occur. These studies highlight the many microglia functions during brain development and the challenge of clearly determining which microglia function is important to a given developmental process in the brain. Additionally, the implications for lifelong brain function and behavior remain unknown in many of these cases.

MICROGLIA AND PSYCHIATRIC DISORDERS OF DEVELOPMENT: SYNAPTIC PRUNING AND BEYOND

As introduced above, microglia are posited to regulate normal and abnormal brain development seen in neuropsychiatric disorders primarily *via* the regulation of synaptic pruning. The strongest evidence that microglia regulate developmental synaptic pruning is the aforementioned studies of the complement cascade in the thalamus. Recently, it was also found that specific complement component 4 (C4) alleles are highly associated with schizophrenia and that C4 knockout in mice resulted in decreased synaptic pruning in the visual thalamus (54). These data support the notion that dysfunction within the complement system may perturb microglia-mediated synaptic pruning and contribute to the onset of neurodevelopmental disorders such as schizophrenia.

An exciting remaining question is how C4 risk variants, or other complement system manipulations, shape behavior relevant to psychiatric disorders in rodents or humans. Recent evidence indicates that C4a is upregulated in the post-mortem tissue of autistic and schizophrenic patients (55). However, C1q and C3 knockout mice only begin to show changes on some tasks when they are aged, although C1q knockout mice do display increased connectivity (56–58). For other regulators

TABLE 1 | Summary of the known major developmental functions of microglia, the ages at which they have been observed, the brain regions in which they have been observed, the major impact of the finding, and the reference associated with the findings. These citations are the basis for the schematic in **Figure 1.**

Developmental function	Age	Brain area	Notable findings	Reference
Synaptic patterning	P12–15	Hippocampus	CX3CR1, social behavior	Paolicelli et al. (12) and Zhan et al. (62)
	P5–9	Barrel cortex	CX3CR1	Hoshiko et al. (11)
	P5	dLGN of thalamus	C3, activity dependent	Schafer et al. (36)
	P2	POA	Sex difference, PGE2, sex behavior	Lenz et al. (33)
	P30+	Motor cortex	BDNF, motor learning	Parkhurst et al. (52)
	P40	dLGN of thalamus	Second wave of synaptic pruning	Schafer et al. (48)
	P15	Spinal cord and thalamus	IL-33m, sensorimotor behavior	Vainchtein et al. (47)
Cell genesis	P2–5	Ventricular area	IL6, IL1B, TNF, IFNY	Shigemoto-Mogami et al. (37)
Myelinogenesis	P6–22	Corpus callosum, cerebellum	IGF1, Cd11c+	Hagemeyer et al. (32)
	P6–22	Corpus callosum	IGF1, Cd11c+	Wlodarczyk et al. (26)
Cellular phagocytosis	E9.5	Neural tube	Earliest noted microglia function	Kierdorf et al. (4)
	P0-2	Hippocampus	Induce cell death, Cd11b	Wakselman et al. (40)
	E17–P6	Cortical proliferative zones	Phagocytosis of progenitor cells	Cunningham et al. (31)
	P4	Hippocampus	VEGFR1, Nox-2	Lelli et al. (41)
	P2–3	Hippocampus	Sex difference	Nelson et al. (28)
Axon dynamics	E14.5	Striatum	Cr3, Dap12/Tyrobp, Cx3cr1	Squarzoni et al. (38)
	E17.5	Corpus callosum	Dap12	Pont-Lezica et al. (35)
Cell positioning	E18.5	Cortex		Squarzoni et al. (38)
Cell survival	P3–4	Cortex	IGF1, Cx3cr1	Ueno et al. (39)

of the complement pathway, such as CUB and Sushi Multiple Domains 1 (CSMD1), an inhibitor of the complement cascade, some studies show they contribute to behavior changes relevant to schizophrenia, whereas others do not (59–61). Recent research indicates that synaptic pruning deficits can alter behavior. IL-33 drives microglia synaptic pruning in the spine and the thalamus, and IL-33 knockout results in altered sensorimotor behavior, but not motor or auditory function (47). The strong association of specific C4 alleles with schizophrenia presents an interesting and unique opportunity to directly connect microglia synaptic pruning with the development of schizophrenia. For example, do specific C4 alleles segregate with specific behavioral/functional impairments (endophenotype/biotype) in humans diagnosed with schizophrenia or another psychotic disorder? If so, microglia synaptic pruning mediated by the complement cascade could be directly connected to specific behavioral/functional impairments in humans.

The data to support the possibility that disruptions in normal microglia-mediated synaptic pruning could contribute to neuropsychiatric disorders are strong so far, yet our previous discussion on the diverse aspects of brain development shaped by microglia is worth keeping in mind. The question that needs to be answered is which of these functions is important for microglia-mediated programming of behavior. Several lines of evidence suggest that we need to be cautious about prematurely concluding that microglia primarily shape brain development and possibly mental health disorders primarily *via* underlying effects on synaptic pruning. For example, disrupting fractalkine signaling during development has been linked to deficits in social behavior that may reflect an autistic-like phenotype (62), and these deficits are posited to occur through dysregulated sculpting of neural circuits (12). Interestingly, however, fractalkine signaling also regulates cortical oligodendrogenesis (63). White matter irregularities are common in people diagnosed with a mental health disorder, including autism and schizophrenia (64–68). Microglia also control axonal fasciculation, which may also contribute to functional connectivity differences between brain regions, including those seen in CX3CR1 knockout mice (35, 38, 62). Microglia are also necessary for spine synapse formation and synapse maturation during development, and this support of spinogenesis and synapse maturation is important for behavior (11, 33, 34, 52). Other cells also contribute to synaptic pruning in the developing brain. Astroglia phagocytize synapses in the dLGN to a greater extent than do microglia (69).

Moving forward, we must consider the behavioral implications of microglia manipulations and attempt to tease out the specific mechanisms through which microglia are influencing behavioral development. With the discovery of microglial-specific genes that are distinct from systemic-macrophage genes, we can begin to use genetic tools to specifically target and manipulate microglia. In this way, we will begin to directly test how microglia influence the development of brain and behavior, whether they do so through synaptic pruning, regulation of myelination or support of neurogenesis, or (as is likely) a combination of all these routes.

THE MICROENVIRONMENT DRIVES MICROGLIA FUNCTION: HETEROGENEITY ABOUNDS

Microglia in the brain are not interchangeable. Peripherally, macrophage function in different tissues is driven by the tissue microenvironment (70, 71). In the adult brain, the microenvironment drives the microglia phenotype in both rodents and humans (72–74). Many factors, such as CSF1, IL-34, TGF-β, cholesterol, CX3CL1, PGE2, and VEGF, are released in discrete brain regions to drive specific microglia functions during development (8, 10, 33, 36, 41, 75). Several studies have shown that TGF-β and CSF1-R signaling are important for microglia development, survival, and identity (6, 72, 76, 77). VEGF, potentially released by neural progenitors, can recruit CD68 + phagocytic microglia during development or induce a phagocytic phenotype (41, 75). Astrocytes seem to be an important mediator of microglia function as well, with astrocyte-conditioned medium supporting microglia survival *in vitro* (72). Interestingly, microglia gene expression is dramatically altered by removing microglia from the brain and culturing them. Culturing microglia increases both inflammatory and developmental genes even in the presence of important astrocyte-derived survival factors (TGF-β, CSF1, and cholesterol) (72). Conversely, cultured microglia rapidly adopt a normal *in vivo* gene expression profile when transplanted into the adult brain (72). Turano et al. (78) showed that microglia inflammatory gene expression in response to immune challenge *in vitro* was different depending on whether other neural cells were also present in culture, suggesting these other cells regulate microglia behavior. There are several neurotransmitters that locally regulate microglia in the adult brain such as nucleotides (ATP, UDP, etc) and histamine (79–81). However, it is unknown how neurotransmitters regulate microglia function during development. These results drive the important point that multiple, locally derived factors regulate microglia identity and function.

The trajectory and specific signaling mechanisms governing brain development are region-dependent (82, 83). However, very little is known about regional differences in microglia during development. We have found that microglia density in the amygdala is higher than it is in the medial prefrontal cortex during the first week of life (84). In contrast, sub-regional differences in microglia function, density and morphology in the basal ganglia do not occur until after 2 weeks of age (73). Similarly, there are regional differences in the timing of Tmem119 expression in the brain, which appears to be a good proxy marker of an adult microglia phenotype (14), suggesting that microglia mature at different rates across the brain. These differences in microglia density and gene expression might reflect the different developmental trajectories of specific brain regions. In addition, as will be discussed further below (see Microglia and Sex Differences: Ignored Phenotypic Differences With Big Implications), there are also prominent sex differences in microglia number, function, and phenotype during brain development. Thus, hormonal signals could also differentially affect the microenvironment during development, depending on regional differences in hormone receptor expression. Together, these results indicate that there are

other uncharacterized CNS intrinsic cues that direct microglia phenotype during development and across tissues.

Alterations in microglia function that result from upstream changes in the CNS environment may also be relevant to understanding the pathophysiology of human psychiatric disorders. Post-mortem brain tissue from individuals with autism is enriched with many gene variants or mutations that are associated with synaptic and neuronal genes, but not microglia-related genes (85–87). However, many genes with altered expression in the same tissue are expressed by microglia and astroglia (85–87). These data suggest that microglia respond to an altered neural environment induced by non-glial risk variants. But the verdict is still out: Recently, conflicting evidence in rodent models of Rett Syndrome studies has arisen. Some evidence suggests that direct microglia dysfunction is implicated in pathology seen in a mouse model of Rett Syndrome (88); other evidence suggests that microglia are downstream responders to an altered brain microenvironment (48, 89). In many cases, it will likely turn out to be not "either/or" but "both"— in other words, microglia may directly drive neuropathology as well as respond to an altered brain microenvironment. The ability of microglia to adapt to the brain environment suggests that microglia function can be built-to-suit, thus generalizations between microglia during development, normal adulthood, or various pathological conditions may be hard to come by.

THE MACRO-ENVIRONMENT DRIVES MICROGLIA FUNCTION

As innate immune cells, microglia are highly responsive to environmental perturbations. We define the macro-environment as large-scale perturbations such as stress or immune challenge that change the microenvironment of the body and brain specifically. Early life perturbations are major risk factors for many psychiatric and neurological disorders, suggesting that brain development is altered by these experiences (90). And yet, there has been very little work that specifically assesses microglia function acutely in the hours, days, and weeks immediately following a macro-environmental perturbation. Most research to date has instead focused on enduring changes in microglia of adults following an early life insult. What research has been performed on acute effects of early life perturbations on microglia has been very interesting. Prenatal or early life immune activation accelerates the maturation of microglia rather than inducing a "pro-inflammatory" phenotype (24, 91). There may also be differences in how microglia function is changed depending on whether immune challenge is initiated using a viral mimetic challenge, such as Poly I:C or a bacterial endotoxin challenge with LPS. Maternal immune challenge with Poly I:C does not seem to induce as much of a "pro-inflammatory" phenotype in microglia compared to LPS (31, 92). However, several studies have found that prenatal Poly I:C and neonatal LPS both accelerate the maturation of microglia (24, 91). Perturbations of the gut microbiome during development can lead to underdeveloped microglia (24, 93, 94). Males and females may also show differential responses to developmental perturbations. For example, male microglia show more dramatic changes in gene networks compared to females when developing in germ-free conditions or after neonatal endotoxin challenge (91, 94). Others have found that prenatal maternal diesel exhaust exposure changes microglia-neuron positioning in the offspring brain, similar to that seen in autism (95, 96). Early life stress is associated with many prominent microglia changes, including downregulation of genes normally expressed in immature microglia, a temporary increase in microglia density in the immature brain, and increased phagocytic activity (29).

Given the heterogeneity in the microglial response to different early life perturbations or timing in these perturbations, it is critical to consider whether the crucial factor that contributes to brain development is altered microglia number (e.g., microglial load), altered microglia function, or both. While many studies have focused on microglia function during development, changes in microglia density could also drive behavioral changes. We and others have found that temporarily depleting microglia during the early neonatal period programmed long-term changes in behavior, including decreased anxiety-like and despair-like behavior, and working memory deficits (84, 97). $CX3CR1^{-/-}$ mice have a transiently decreased microglia density in the hippocampus and delayed microglia entry into the barrel cortices and social behavior (11, 12, 62). In contrast, microglial loss in adulthood seems to have little impact on behavioral outcomes (77). It is important to note that the changes in microglia density are temporary and need not be permanent to have lasting effects. Changes in microglia density could mean that there is a change in the magnitude of microglia's influence on brain development (e.g., fewer microglia, less synaptic pruning), without overt changes in microglia function. Conversely, changes in microglia density could be compensated for by changes in microglia function (e.g., more microglia, less synaptic pruning per microglia) or vice versa. Changes in density due to prenatal/early life challenges could also reflect changes in maturation of microglia. We must begin to determine the functional and behavioral changes that occur following specific targeted microglia manipulations to significantly advance the translational impact of the field of developmental neuroimmunology.

MICROGLIA AND SEX DIFFERENCES: IGNORED PHENOTYPIC DIFFERENCES WITH BIG IMPLICATIONS

During ontogeny, the brain is permanently organized as male- or female-typical in a process called sexual differentiation. Sex-specific brain development supports the emergence of behaviors necessary for reproduction, parenting, and social behaviors such as aggression [reviewed in Ref. (98)]. Sexual differentiation is driven by sex-specific hormonal signals. In mammals, the major hormonal signal is the androgen testosterone, which is secreted by the fetal testes (99). In human males, testosterone secretion occurs prenatally, and this testosterone enters the developing brain and binds to androgen receptors, which act as transcription factors to shape sex differences in gene expression. In humans, the process of sexual differentiation is largely completed by birth. In rodents, the major elements of sexual differentiation are the same, with several notable exceptions. Unlike in humans, the rodent testis begins secreting testosterone on E18, 3 days prior to birth, and testosterone secretion ends during the first postnatal day (100). However,

the critical period in which the brain remains sensitive to the early programming effects of hormones extends until approximately P10. Also unlike humans, in rodents, testosterone is converted into another steroid hormone, 17-beta estradiol, in the brain by the enzyme p450 aromatase, and estradiol binds to estrogen receptors in the brain to effect male-typical brain organization (101, 102). In females (both human and rodent), the process of sexual differentiation proceeds along a "default path" in the absence of an active hormonal signal (103), but also has a similar critical period.

Many of the major processes of brain development proceed differently in males and females, including cell genesis, cell death, cell migration, axon guidance, synaptic patterning, and myelination (see McCarthy et al. (104) for thorough review). Sex differences in both microglia number and their properties have been documented in the developing brain. We have thoroughly reviewed these sex differences in microglia and inflammatory mediators elsewhere, both in the context of normal brain development as well as in response to early life perturbations [see Nelson and Lenz (105).]. Nevertheless, several important points are worth repeating, especially given the recent National Institutes of Health mandate that all studies consider the contribution of biological sex to their results.

In many brain regions, males have more microglia in the developing brain and tend toward a more ameboid morphology (25, 33). This may indicate either greater activation of microglia in males or possibly a more immature phenotype (25, 33, 91). Indeed, female microglia appear to mature and reach an adult phenotype earlier in development than do male microglia (91). There are also sex differences in microglial phagocytosis in the rat hippocampus, with females having higher levels of phagocytosis and phagocytic gene expression than males (28). In this case, female microglia engulf neural progenitor cells at higher rates than in males (28), suggesting that microglia could regulate a known sex difference in neurogenesis in the developing hippocampus (106). Sex differences in microglia are not present in the rodent brain prior to the onset of the testicular androgen surge, but are seen soon after the surge occurs (25). Treating females with male-typical hormones (estradiol or testosterone) during the critical period for sexual differentiation induces a male microglia phenotype within days, indicating microglia are responding to steroid hormones (28, 33). However, in both our work and that of other research groups, steroid hormone receptor expression is either extremely low or undetectable in microglia in the developing brain (33, 78, 107). This suggests that crosstalk between microglia and other steroid-sensitive cells is necessary for microglia sexual differentiation to occur. It remains to be determined how the sex differences in microglia number are programmed. Differential chemotactic signals attracting microglia to particular brain regions are one possibility (25). Another possibility is differential proliferation or cell death, yet we have not seen sex differences or hormonal regulation of microglia proliferation in the developing hippocampus (28).

Not only are microglia targets of the sexual differentiation process, but they are also key effectors of sexual differentiation. Many of the mechanisms through which sexual differentiation occurs (e.g., cell genesis, cell death, synaptic patterning, and myelination) are regulated by microglia. Thus, a natural question is whether microglia contribute to the sexual differentiation process. Although this question has only been addressed in select few studies, it does appear to be the case. In the rodent preoptic area (POA), there is a sex difference in synaptic patterning that is organized by exposure to neonatal androgens, such that neurons in the male POA have two to three times the number of dendritic spine synapses than do neurons in the female POA (108, 109). A higher level of dendritic spines in the male POA persists until adulthood, and the number of dendritic spines correlates positively with the number of male-typical mounting behaviors in adult mating tests (109, 110). This developmental sex difference depends upon estradiol, as well as its downstream effector, the inflammatory molecule prostaglandin E2 (PGE2). If female rodents are treated with either estradiol or PGE2, they will show male-typical dendritic spine density in the POA as well as male-typical mounting behavior in adulthood (109). Microglia are necessary players in this process in the POA. Estradiol-induced masculinization of PGE2 levels, dendritic spines as well as male-typical adult sexual behavior can be blocked by concurrent administration of the microglial inhibitor, minocycline, during development (33). Temporary ablation of microglia from the developing brain leads to diminished performance of males, though not females, on sexual behavior tests in adulthood (97).

Another study of the nearby anteroventral periventricular nucleus (AVPV) of the POA also suggests immune regulation of sexual differentiation. The AVPV is smaller in males due to hormonally induced acceleration of cell death in the developing male, and in females, the nucleus is responsible for adult hormonal cycling (111). In the AVPV, a sex difference in the immune factor TNF family member repressor protein (TRIP) drives this sex difference (112). The cellular source of this immune factor has not been determined, but may well be microglia. The previously described sex differences in microglia properties in the immature brain suggest that many more such instances are yet to be uncovered in which microglia contribute directly to sexual differentiation of brain and behavior.

In rodents, diverse challenges such as early life bacterial infection (113), prenatal high fat diet (114), intrauterine inflammation (115), early life stress (116–118), and prenatal exposure to diesel particulate (95) all elicit either microgliosis or increased microglia number in the developing offspring brain. In response to these varied early life perturbations, male rodents tend toward greater microglial reactivity in the brain as well as greater inflammatory gene expression than females. In addition, transcriptome profiling shows that challenge with LPS accelerates microglial maturation index in males, but not females, again suggesting that males are more vulnerable to inflammatory insults during this period (91). This same study used transcriptome data analysis of human brain samples and found that the microglial developmental index was accelerated in postmortem tissue from individuals with autism spectrum disorder (ASD) and Alzheimer's, indicating that this sex-specific acceleration of microglia maturation may be relevant to sex differences in human disease pathophysiology. Microglia from germ-free mice also show a sex difference during development and in adulthood (94). Male microglia show more gene expression changes during development whereas female microglia show more gene expression changes during adulthood in response to a loss of intestinal microbes. Given that two different developmental perturbations resulted in more changes in male microglia, it will be interesting to see whether this same sex difference in microglia is generalizable to other developmental perturbations.

In the context of human neurodevelopmental disorders, sex differences in microglia or other immunocompetent cells may be central. Many brain-based disorders of development, including autism, attention deficit hyperactivity disorder (ADHD), Tourette disorder, and schizophrenia [reviewed in Ref. (104)]. Autism is one of the most sex-biased disorders, though the reasons for this sex bias are unknown. Early life inflammatory experiences increase the risk for neurodevelopmental disorders (119–123), thus immune mediators may contribute to this sex bias. A study of postmortem brain tissue from autistic individuals has shown that there are sex differences in astrocyte and microglia markers in post-mortem autistic brain, but not in autism risk genes (124). This means that sex differences in autism risk gene expression are not likely responsible for the higher rate of autism in males. Instead, it suggests that microglial and astrocytic genes are more highly expressed in the male brain independent of autism risk genes to increase risk for autism and possibly other comorbid disorders of brain development. However, others have found that, while isolated murine microglia show a sex difference during development, isolated human microglia do not (94). Further research is needed to determine whether microglia contribute to the sex differences in human neurodevelopmental disorders.

WHAT ABOUT MICROGLIA IN HUMANS?

A majority of research on microglia has been performed in rodent models, and to date, relatively little research has been done to determine whether microglia function is similar in humans. Several commentaries have addressed the differences between murine and human microglia and the immune system [see reviews (125–127)]. Here, we highlight the important differences and similarities in microglia function across species during development as well as analysis of microglia in neurodevelopmental disorders. Only 30% of microglia genes in humans are enriched in mouse microglia (128); however, there is a core set of important microglia genes whose expression is conserved between humans and mice (94). There are several differences in molecules that regulate phagocytosis such C4a, C4b, and Siglec-11 which are present in humans, but not mice (mice do express a single C4 isoform), and Siglec-H and Fcrls which are present in mice, but not humans (54, 76, 129, 130). Studying the functional impact of genes that are either differentially expressed or not expressed at all in one or the other species will likely prove challenging, especially when these differences are highly associated with neurodevelopmental disorders. However, there are some crucial similarities that justify continued use of rodent models to study microglia function during development. Microglia colonize the human brain over a similar timeline to that in rodents (2, 131). Microglia phagocytize progenitors in mice and primates in the ventricular areas and exhibit similar increases in phagocytic microglia as the progenitor proliferation begins to decrease (31). We also know that microglia function is similarly shaped by the microenvironment, even if the microenvironment is slightly different between humans and rodents (19, 24, 74). With the development of new and better tools, we can likely identify where the crucial differences are and how to account for them experimentally.

Neuroinflammation in human neurological or neuropsychiatric disorders has been assessed using PET scanning with a ligand to visualize the translocator protein (TSPO) on microglia in the brain (132). Such studies have shown increases in putative microglia activation in many neuropsychiatric disorders, including autism and major depression (133, 134). These studies certainly indicate a change in glia, but alone do not indicate how microglia function is altered in these conditions (132). For example, TSPO signal has been seen to decrease in schizophrenia despite other evidence of neuroinflammation in the condition (135). In addition to microglia, TSPO is expressed in activated astrocytes, and TSPO expression is not solely affected by inflammation, but rather by the specific microenvironment (135). Since these differences in TSPO binding in neuropsychiatric disorders likely mean some change in glial function has taken place, future research should focus on more in-depth analysis to assess the specific functional changes in microglia (or astrocytes) in these disorders.

We also know very little about what microglia are doing during development in humans who go on to develop a psychiatric disorder later in life. Part of the challenge is that many changes in microglia function and related brain development are likely to occur well before symptoms are present. Several postmortem studies have found increased number and reactivity of microglia in developmental disorders, including autism, schizophrenia, and Tourette disorder (136–139). Transcriptome analysis of post-mortem tissue from individuals with autism and Tourette disorder shows that the differentially expressed genes are highly enriched in microglia and/or immune genes (124, 138). It should be noted that not all studies of post-mortem tissue have found dramatic changes in microglia density or morphology (137, 139). Additionally, it has not been determined whether microglia changes observed within the postmortem brains of individuals with neurodevelopmental disorders are part of the etiology and pathophysiology of the disorder or instead a downstream response to a dysfunctional or deteriorating brain environment.

Despite these caveats, several pieces of data converge to make a strong case that microglia dysfunction is actively contributing to the pathophysiology of neurodevelopmental disorders. First, the previously discussed schizophrenia study implicating a complement system risk variant in the disorder (54); second, the Rett syndrome study showing that microglia manipulations are therapeutically effective in the rodent model of the disorder; and third, the autism study showing higher expression of glial genes in males with autism (124). Fourth, a very recently published paper shows substantial overlap in neuronal gene modules associated with several neuropsychiatric disorders, but that autism is associated with a unique upregulation in a glial-related gene expression module (55). What is more, the microglia modulate upregulated in ASD includes IRF8, a transcription factor important for the transition from pre-microglia to early microglia, which suggests that the development of microglia may be altered in ASD (4, 55).

OTHER IMMUNOCOMPETENT CELLS IN THE BRAIN: IN CONVERSATION WITH MICROGLIA?

Microglia are by far the most abundant immune-derived cell type in the brain. Yet other innate immune cells have also been detected within the healthy developing brain. Of note are mast

cells, which are tissue-resident innate immune cells that are similar to basophils. Mast cells have been detected in the rodent and human brain under healthy conditions. Interestingly, in the healthy human brain, mast cells are most often detected within the developing brain (140) but their function is largely unknown. In mice, mast cells are detectable throughout the lifespan (141). Mast cell-deficient mice show several abnormalities that suggest their function is crucial in the developing or adult brain. For example, mast cell deficient mice show deficits in learning and memory, hippocampal neurogenesis, and increased anxiety-like behavior in adulthood (141, 142). Mast cells are a potent source of amine neurotransmitters such as serotonin and histamine (143) and may function as neuromodulatory cells. However, mast cells also release a host of inflammatory molecules, including cytokines, chemokines, and prostaglandins (143), all of which can regulate brain function in healthy or inflammatory conditions. Mast cells may also regulate microglial activation *via* secretion of these mediators, with recent *in vitro* studies showing that conditioned mast cell medium can induce the release of pro-inflammatory cytokines in cultured microglia (144). Antagonizing histamine receptors, proteinase activated receptors, and toll like receptor (TLR) 4 prevented these effects on microglia, suggesting that multiple mast cell mediators may be involved in mast cell-microglia crosstalk. Future studies are necessary to determine how crosstalk between different immune cell types occurs *in vivo* and whether this crosstalk is important for normal brain development or abnormal development following early life perturbations.

Astrocytes also make up a huge proportion of cells within the brain. Although derived from neural stem cells in neurogenic niches in the brain (145), astrocytes are immunocompetent insofar as they release and respond to immune system mediators (such as cytokines) and are capable of antigen presentation [reviewed in Dong and Benveniste (146)]. But what is the nature of crosstalk between microglia and astrocytes? In neurodegenerative studies, crosstalk between the two cell types has been well demonstrated, often with activated microglia inducing a neurotoxic phenotype in astrocytes (147). Environmental perturbations that lead to altered microglial gene expression and microgliosis in the brain, such as immune challenge with LPS, also induce the release of proinflammatory mediators from astrocytes (148, 149). Interestingly, astrocytes also prune synapses during development, suggesting that microglia could indirectly change synaptic pruning by releasing cytokines or other signals that alter astrocyte function (69, 147). What has been largely lacking to date in developmental neurobiology research is any careful investigation of chicken-and-egg relationships, including specific signals between microglia and astrocytes that regulate their function in either direction. A very recently published article, however, has taken an important step in this direction, showing that astrocyte-derived IL-33 drives microglia phagocytic activity in the developing central nervous system (47).

While the healthy brain does not contain large quantities of peripherally derived immune cells, recent evidence nevertheless suggests that peripheral cells can influence normal brain function. One potential route is *via* effects in the meninges that are conferred across the blood brain barrier. Adaptive immune cells, such as T cells, are present within the meninges, and they regulate brain function and the display of social behavior *via* interferon signaling across the blood brain barrier (150). T cells have also been implicated in brain and behavioral development, particularly in sexual differentiation. T cell-deficient mice display altered size of several areas of the brain, including the hypothalamus, amygdala, periaqueductal gray, and raphe nuclei (151). Additionally, T cell-deficient animals have a loss of sexual dimorphism in the size of several brain regions, such as the bed nucleus of stria terminalis, with females resembling wild-type males (151). Finally, these animals show decreased anxiety, suggesting that T cells are necessary for programming of mood related behavior. The mechanisms through which T cells influence brain and behavioral development are yet to be determined, though it may well be that T cell-derived signaling across the blood brain barrier influences microglia function during the critical period for brain organization.

CONCLUSION AND FUTURE QUESTIONS

As with any young field, it is important not to conclude too early that we truly understand how microglia shape brain development. What we do know is that microglia make up a significant percentage of cells in the brain throughout life and that they are as important in healthy conditions as they are in pathological conditions. We have attempted to move beyond summarizing the role microglia are known to play in brain development and function in order to point out that in many cases, we know very little and much more basic discovery work is needed. We highlight the diversity of microglia function in the developing brain to emphasize their potential importance to understanding and treating brain-based disorders of development. At this stage, we have few sophisticated tools to manipulate microglia function beyond cell-type specific knockout models. Thus a future goal of the field should be to develop new tools to manipulate microglia function, both phagocytic function and release of secreted factors, in targeted ways to connect microglia behavior to structural, functional, and behavioral outcomes in living organisms. It is an exciting time to be researching neuroimmune function, and the promise that microglia could be viable targets to prevent or treat brain based disorders is high.

AUTHOR CONTRIBUTIONS

KL and LN planned and wrote the manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Phosphatidyl-Inositol-3 Kinase Inhibitors Regulate Peptidoglycan-Induced Myeloid Leukocyte Recruitment, Inflammation, and Neurotoxicity in Mouse Brain

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Acute brain injury leads to the recruitment and activation of immune cells including resident microglia and infiltrating peripheral myeloid cells (MC), which contribute to the inflammatory response involved in neuronal damage. We previously reported that TLR2 stimulation by peptidoglycan (PGN) from *Staphylococcus aureus*, *in vitro* and *in vivo*, induced microglial cell activation followed by autophagy induction. In this report, we evaluated if phosphatidyl-inositol-3 kinase (PI3K) pharmacological inhibitors LY294200 and 3-methyladenine (3-MA) can modulate the innate immune response to PGN in the central nervous system. We found that injection of PGN into the mouse brain parenchyma (caudate putamen) triggered an inflammatory reaction, which involved activation of microglial cells, recruitment of infiltrating MC to injection site, production of pro-inflammatory mediators, and neuronal injury. In addition, we observed the accumulation of $LC3B^+$ $CD45^+$ cells and colocalization of LC3B and lysosomal-associated membrane protein 1 in brain cells. Besides, we found that pharmacological inhibitors of PI3K, including the classical autophagy inhibitor 3-MA, reduced the recruitment of MC, microglial cell activation, and neurotoxicity induced by brain PGN injection. Collectively, our results suggest that PI3K pathways and autophagic response may participate in the PGN-induced microglial activation and MC recruitment to the brain. Thus, inhibition of these pathways could be therapeutically targeted to control acute brain inflammatory conditions.

Keywords: myeloid cells, phosphatidyl-inositol-3 kinase inhibitors, autophagy, peptidoglycan, TLR2, inflammation

INTRODUCTION

Regulation of immune response in the brain is in part accomplished by myeloid cells (MC), which represent a diverse group of mononuclear phagocytic cells (1–3). The healthy central nervous system (CNS) parenchyma contains only one type of MC, the parenchymal microglial cell, which are tissue-resident macrophage confined in an immunosuppressive environment (4, 5). However, during parenchymal brain injury, microglial cells become activated and mediate

the local immune response. These cells are a prominent source of pro-inflammatory factors and oxidative stress mediators, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, chemokines, nitric oxide (NO), all of which are further neurotoxic (6, 7). In response to tissue damage, infiltrated blood-borne MC migrate to the affected area and may contribute the neuroinflammatory process (8). Nevertheless, the differential roles of these MC populations in CNS disorders have only recently been acknowledged.

Toll-like receptors (TLRs) are a family of pattern-recognition receptors in the innate immune system. Exogenous and endogenous TLR ligands activate microglia that trigger inflammatory reactions in CNS (9–11). Recent studies using a mouse experimental brain abscess model have revealed a complex role for TLRs in the disease pathogenesis (12). Interestingly, TLR2 participates in the innate immune response during the acute stage of brain abscess formation induced by *Staphylococcus aureus* and influences adaptive immune response (13, 14). Moreover, MyD88, a central adapter molecule for many TLRs including TLR2, is a key component in the brain innate immunity and was involved in exaggerated brain tissue destruction (15, 16). Numerous reports suggest that TLR2 contributes to the phagocytosis and autophagy in professional phagocytes upon bacterial infection (17–19). In line with such evidences, we previously demonstrated that intracerebral delivery of peptidoglycan (PGN), the major surface component of Gram-positive bacteria and a potent TLR2 agonist, leads to the autophagy activation in microglial cells *in vivo* (20). In addition, we observed that both, 3-methyladenine (3-MA) and LY294002 inhibited autophagy activation in microglial cells and reduced NO production.

Autophagy is a conserved process, whereby cells deliver cytoplasmic contents to the lysosomes for removal (21). Although this mechanism has a main protective function (22), under several conditions, deregulated autophagy may contribute to the inflammation and tissue injury (23). Enhanced autophagy has been implicated in various neurological conditions including intracerebral hemorrhage, cerebral ischemia, and spinal cord injury (24, 25). This self-degradation process is emerging as a core regulator of CNS inflammation, aging, and neurodegeneration (26). In the brain, it has mostly been studied in neurons, where the delivery of toxic molecules and organelles to the lysosomes by autophagy is crucial for the neuronal health and survival (27, 28). The initiation and course of the autophagic flux are regulated by Beclin 1/Class III phosphatidyl-inositol-3 kinase (PI3K)-containing complexes (29). Nevertheless, PI3K have dual role in autophagy induction since, class III PI3K is required for the autophagosome formation but class I PI3K interact with principal negative regulator of autophagy mTOR (30).

PI3Ks regulate several key events in the inflammatory response to damage and infection and they were implicated in the regulation of the pro-inflammatory responses induced by TLR activation (31). However, the role of PI3Ks in the signaling pathways downstream of TLRs on MC is not completely clear. Different studies suggested that this pathway can play either positive or negative roles in the production of pro-inflammatory cytokines (32–34). Furthermore, most of the studies were performed in the peripheral immune system, and little is known about the contribution of the PI3K activity and autophagy in the regulation of the neuroinflammatory response elicited by PGN.

We propose that the inhibition of PI3K and autophagy could modulate CNS inflammation induced by TLR2 stimulation. Therefore, the aim of this study was to evaluate if intracerebral administration of PI3K inhibitors could regulate neuroinflammatory responses induced by PGN. We observed that this TLR2 ligand induced MC activation and colocalization of LC3B and lysosomal-associated membrane protein 1 (LAMP1) molecules in the CNS. In addition, PI3K inhibitors (including 3-MA) prevented the recruitment of inflammatory MC to the brain and reduced signs of neurodegeneration. Furthermore, here we show that PI3K inhibitors differentially regulated the PGN-elicited production of pro-inflammatory molecules and chemokine receptor expression in brain MC. Our findings suggest that induction of neuroinflammation by PGN was TLR2-dependent and it may require PI3K activation and autophagy. Inhibition of these pathways in the brain may lead to the downregulation of both, microglial cell activation and leukocyte recruitment to CNS, resulting in neuronal protection.

MATERIALS AND METHODS

Reagents and Animals

Peptidoglycan from *S. aureus* and 3-MA were purchased from Sigma-Aldrich (St. Louis, MO, USA). LY294002 were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). In this study, 6- to 8-week-old male C57BL/6J, TLR2 KO, or MyD88 KO mice were used. C57BL/6J mice were purchased from the Facultad de Ciencias Veterinarias, Universidad Nacional de La Plata, Argentina; C57BL/6 TLR2-knockout and C57BL/6 MyD88 knockout mice were purchased from The Jackson Laboratory, Bar Harbor, ME, USA. Animal care was provided in accordance with the procedures outlined in the "Guide for the Care and Use of Laboratory Animals" (NIH Publication No. 86-23, 1985). The experimental protocols were approved by the Institutional Animal Care and Use Committee of Centro de Investigaciones en Bioquímica Clínica e Inmunología (CIBICI), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET). Our animal facility obtained NIH animal welfare assurance (assurance number A5802-01, OLAW, NIH, USA).

Surgical Procedures

After 1 week of acclimatization to the housing facility, 6- to 8-week-old male C57BL/6J mice were anesthetized with a combination of ketamine/xylazine. The mouse scalp was shaved and scrubbed with hydrogen peroxide. Animals were placed in a Thomas stereotaxic frame (Philadelphia, PA, USA). A midline incision was made, the skin was retracted, and one small bore hole was drilled into the skull. The infusion cannula (30 G; 20 mm) was stereotaxically lowered into the caudate putamen (CPU) using the following coordinates: anterior, +0.8 mm; lateral, +1.5 mm; ventral, −3.2.0 mm, according to the atlas of Franklin and Paxinos (2008). The infusion cannulae were connected *via* polyethylene tubing (PE 10; Becton Dickinson, Sparks, MD, USA) to 10 µl microsyringes (Hamilton, Reno, NV, USA) mounted on a

microinfusion pump (Harvard Apparatus, Holliston, MA, USA). Each mouse was injected with 5 µg PGN alone or in combination with 0.19 nmol LY2942002 or with 6.37 nmol 3-MA at a volume 0.35 µl/side at a flow rate of 0.35 µl/min. This volume was selected according to the size and structure of these nuclei. Immediately after the microinjection, the cannulae were retracted, the holes were covered with wax, and the skin was sutured with surgical thread. At different time after surgery, mice were killed, and brains processed for analysis.

FACS Analysis

For *ex vivo* analysis, at different time points, the mice brains were rapidly removed and placed on ice in an acrylic brain matrix (Stoelting Co., USA). Coronal brain slices of 2.0 mm containing the CPU from each hemisphere were dissected. The rest of brain tissues (BRAIN) were collected separately of CPU section, then all these sections were homogenized with scissors on ice and whole brain cells were costained with anti-CD45 (APC-Cγ7) anti-CD11b (PerCPe) antibodies, Ly6C (PE-Cγ7), IA/IE (APC), CD86 (PE), CX3CR1 (FITC), and CXCR4 (APC) (Becton Dickinson, San Jose, CA, USA). Stained cells were analyzed by flow cytometry on a FACSCanto II cytometer (Becton Dickinson, San Jose, CA, USA), using FACS DIVA™ software V 6.0, and data analysis was conducted using FCS express (*De Novo* Software).

Staining of single-cell suspension of isolated brain immune cells was performed using standard protocols. Briefly, cells were stained for surface markers for 30 min at 4°C and washed twice before analysis. All staining procedures were completed at 4°C in DPBS containing 5 mM EDTA and 1% FCS. For all assays, the frequency of resident myeloid cells (RMC) or microglial cell was determined by flow cytometry gating on $CD45^{low}CD11b^{+}$ cells since, the frequency of infiltrating myeloid cells (IMC) is corresponding to $CD45^{high}CD11b^{+}$ cells. Furthermore, the frequency of monocytes is corresponding to $CD45^{high}CD11b^{+}LY6C^{+}$ cells.

Fluorescence Confocal Microscopy

For tissue fluorescence confocal microscopy, at different time points after surgery, mice were anesthetized, perfused with PBS, and then with 4% paraformaldehyde, sacrificed and the whole brains were obtained. After treatment with sucrose, brain tissues were cut on a freezing sliding microtome at a thickness of 10 µm (Shandom Cryotomo E, Thermo Fisher Scientific). The sections were mounted in adhesive slides (KNITTEL StarFrost® slides, Germany) and were then incubated with 5% normal goat serum (Sigma) in PBS, 0.05% Tween-20 (PBS-T-NGS), for 1 h to reduce nonspecific binding of antibodies to the cell surface and for cell permeabilization. An anti-LC3B (Cell Signaling Technology Beverly, MA, USA) plus anti-LAMP-1 (Abcam, Cambridge, UK) or plus anti-CD45 (Biolegend, San Diego, CA, USA) antibodies were applied to the slides, which were further incubated for 1 h at room temperature. After three rinses with PBS, the slides were incubated with Alexa Fluor 488 plus Alexa Fluor 546 secondary antibodies (Life Technology, Carlsbad, CA, USA) for 60 min. The slides were analyzed under a laser scanning confocal fluorescence microscope (Olympus FV300, Tokyo, Japan). In this experimental procedure, the injection sites were defined as the last section containing a visible needle track and the next section without the needle artifact. Quantification of $LC3B^{+}$ microglial cell numbers and number of vesicles LC3B/LAMP-1^{+} cells in brain slides was performed using the software ImageJ (NIH, USA).

Detection of Neuronal Degeneration

Neuronal degeneration was analyzed by the Fluoro-Jade B (FJB) techniques. In a first step, slides were staining with anti-NeuN antibody following staining procedures previously described. Briefly, slides were first immersed in a solution of 0.06% potassium permanganate for 10 min. The slides were then rinsed in distilled water for 2 min. The staining solution was prepared from a 0.01% stock solution of FJB (Chemicon) according to the manufacturer's instructions. To make up staining solution, stock solution was added to 0.1% acetic acid vehicle. This resulted in a final dye concentration of 0.0004% prepared within 10 min of use and was not reused. After 20 min in the staining solution, the slides were rinsed for 1 min in each of three distilled water washes. The slides were then placed on a slide warmer, set at approximately 50°C, until they were fully dry. The dry slides were cleared by immersion in xylene for at least 1 min before coverslipping with DPX (Fluka, Milwaukee, WI, USA; or Sigma Chem. Co., St. Louis, MO, USA). The tissue was then examined using a laser scanning confocal fluorescence microscope (Olympus FV1000, Tokyo, Japan).

Real-Time PCR

Gene expression for iNOS, IL-1β, IL-6, TNFα, CCR2, and CCL2 were assessed using semiquantitative real-time PCR. Briefly, RNA was isolated from CPU brain cells using a single-step phenol/chloroform extraction procedure (Trizol; Life Technologies, Carlsbad, CA, USA) and depleted of contaminating DNA with RNase-free DNase kit (Life Technologies) before reverse transcription. Reverse transcription was performed on 1 µg of RNA using random hexamers as primers by the high-capacity cDNA RT kit (Life Technologies). Real-time PCR was performed by using One step plus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Briefly, 5 ng of reverse-transcribed cDNA was used in triplicate samples. The assays were initiated with 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. Primers were obtained from Applied Biosystems (Foster City, CA, USA). Detection of all target genes and control HPRT was performed using SYBR® Green expression assays Master Mix (Applied Biosystems) and relative quantification (RQ) was calculated by using StepOne™ software V2.2.2 and the $RQ = 2^{-\Delta\Delta Ct}$ method, where Ct is the threshold cycle to detect fluorescence. Ct data were normalized to the internal standard HPRT. The primers sequences used are listed in **Table 1**.

Statistical Analysis

All experiments were performed at least three times and the results presented are from representative experiments. Statistical analyses were performed using either Student's *t*-test for two-group comparison, one-way ANOVA followed by Bonferroni *post hoc* test for more than two groups a one factor or two-way ANOVA followed by Bonferroni *post hoc* test for more than two factors. In all the experiments, $p < 0.05$ was considered to be statistically significant.

TABLE 1 | Real-time PCR primers.

Gene	Real-time PCR primers sequence (5′–3′)
mHPRT1	Sense TCAGTCAACGGGGGACATAAA Antisense GGGGCTGTACTGCTTAACCAG
mCCR2	Sense GTATCCAAGAGCTTGATGAAGGG Antisense GTGTAATGGTGATCATCTTGTTTGGA
mCCL2	Sense CCCACTCACCTGCTGCTACT Antisense TCTGGACCCATTCCTTCTTG
mTNF-α	Sense AGCCGATGGGTTGTACCTTGTCTA Antisense TGAGATAGCAAATCGGCTGACGGT
mIL-1β	Sense ACAGAATATCAACCAACAAGTGATATTCTC Antisense GATTCTTTCCTTTGAGGCCCA
mIL-6	Sense ATCCAGTTGCCTTCTTGGGACTGA Antisense TAAGCCTCCGACTTGTGAAGTGGT
miNOS	Sense GTTCTCAGCCCACAATACAAGA Antisense GTGGACGGGTCGATGTCAC

RESULTS

PGN Injection Induces an Increase in the Frequency of MC in Mouse Brain Parenchyma

We have previously shown that PGN from *S. aureus* is a potent activator of BV2 microglial line cell (20). To examine *in vivo* if PGN is able to promote activation of resident microglial cells in mouse brain (35), we stereotaxically injected PGN into brain parenchyma (CPU). Microglial cells were analyzed in nervous tissue slices by confocal immunofluorescence microscopy. Staining brain sections with CD45 allowed us to distinguish MC ($CD45^+$) from astrocytes, oligodendrocytes, neurons ($CD45^-$), and also $CD45^{bright}$ hematogenous population from $CD45^{dim}$ resident microglia. In brains from PBS-injected mice, microglia showed resting morphological features (36, 37), such as radial, non-overlapping processes, a small cell soma and each cell appearing to occupy its own domain (**Figure 1A**). By contrast, after PGN injection, we observed rounded cells with enlargement of the soma, retraction and shortening of cell processes, resembling amoeboid microglia (**Figure 1A**). These morphological features are suggesting that PGN induce microglial activation (38), which agrees with our previous *in vitro* data.

Central nervous system damage commonly entails recruitment of circulating immune cells, resulting in an innate immune response that consists of resident microglia and peripherally derived monocytes, macrophages, and dendritic cells (8, 39, 40). Thus, we wanted to determine whether PGN increased MC numbers into the brain parenchyma. After PGN injection, CPU was separated at different time points (24 h, 48 h, 72 h, 7 days, and 14 days) and analyzed for the presence of MC by flow cytometry. As expected, PGN resulted in a significant increase in the number of MC in all time points assessed; reaching a peak of cells recruited 72 h after injection (**Figure 1B**). These results correlated with the observation by confocal microscopy that the number of $CD45^+$ cells, in the vicinity (next three sections without the needle artifact) of the site of injection, increased after the administration of PGN (**Figure 1C**). Then, to know whether PGN-induced leukocyte recruitment mostly confined to the vicinity of injection, we examined the injection site (ipsilateral-CPU), the opposite non-injected CPU control (contralateral-CPU), and the whole brain (Brain). We identified tissue RMC versus IMC by flow cytometry, using anti-CD45 plus anti-CD11b antibodies (20). PGN injection strongly enhanced the recruitment of inflammatory cells in the CPU, compared with controls (**Figures 1D,E**). In contralateral-CPU and the whole brain, PGN also increased the number of MC, but in a weaker manner (**Figures 1D,E**). The increase in the number of MC was mainly due to a rise in the number of $CD11b^+CD45^{high}$ fraction (IMC) in brains of PGN-injected mice (**Figures 1D,F**). In addition, we also observed an increase of RMC only confined to ipsilateral-CPU (**Figures 1D,G**). In agreement with the changes described in the literature for acute injured CNS (1, 41), in these experiments we found that PGN-induced microgliosis, microglial cell activation, and recruitment of IMC in brain tissue.

Autophagy Requirement for the Recruitment of MC to Brain Parenchyma After PGN Injection

We previously demonstrated that intracerebral injection of PGN increased autophagy of microglial cells (20). Atg8/LC3 is the most widely monitored autophagy-related protein, and it was originally identified as a subunit of microtubule-associated protein 1 light chain 3 (42). The induction of autophagy in PGN-injected mice was monitored by morphometric analysis after the formation of LC3B-labeled autophagosomes (≥1 μm) (43, 44). In this study, we confirmed that PGN induced an increase in the number of $LC3B^+$ puncta in $CD45^+$ cells, in the brain parenchyma, compared with control (**Figures 2A,B**). In addition, PI3K inhibitors, such as LY294002 and 3-MA, that block class I as well as class III PI3Ks, prevent autophagosome formation, and finally suppress autophagy (23, 45), were able to prevent the increase of LC3B punctate parenchymal MC by PGN treatment (**Figures 2C–F,I**). Moreover, we next examined whether PGN induces convergence of the autophagic pathway with a functional degradative compartment. Staining brain sections with antibodies against LC3B plus the LAMP1 allowed us to visualize the fusion of autophagosomes with lysosomes by confocal microscopy (46, 47). Similarly, to our previous report (20), PGN injection into the CPU significantly induced overlapping signals of LC3B and LAMP1 compared with control, suggesting the autophagic flux is taking place (**Figures 2G,H,J**).

Next, we determined whether the presence of 3-MA and LY294002 affected inflammatory recruitment in the CPU of PGN-injected mice. Wild-type (WT) mice were injected with PGN plus 3-MA or PGN plus LY294002, 72 h later, the number of MC was analyzed by flow cytometry. Interestingly, we found that both autophagy inhibitors, 3-MA or LY294002, decreased the number of MC in the CPU of PGN-injected mice (**Figure 3A**), and this effect was mostly confined to MC in the ipsilateral side, since the analysis of the whole brain, showed that both inhibitors failed to modify the number of cell populations (**Figure 3B**). In addition, here we reveal that these autophagy inhibitors decreased the number of IMC cells in the PGN-injected parenchymal mice but increased the number of RMC (**Figures 3C,D**). Similar effects of

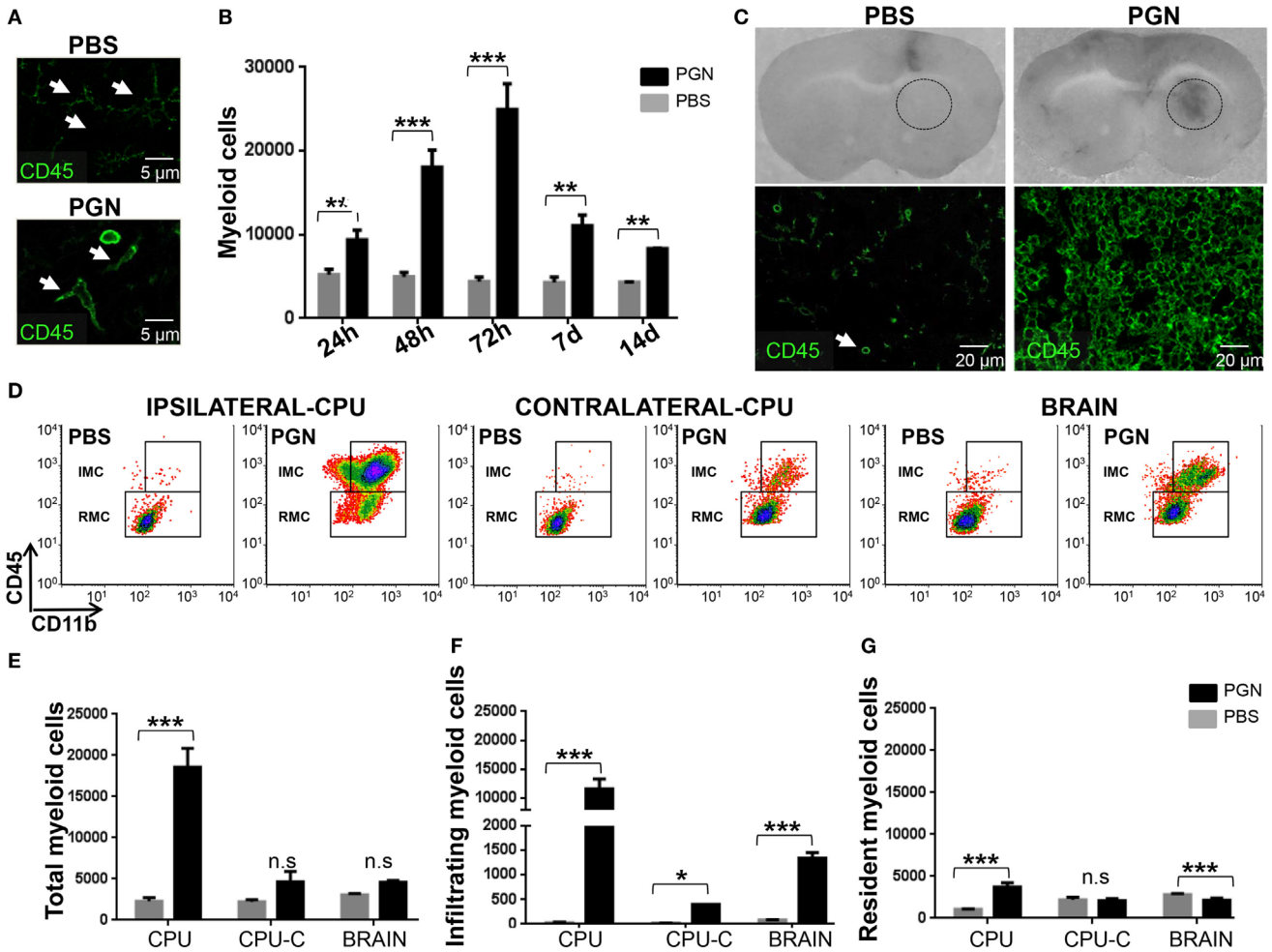

FIGURE 1 | PGN injection induces myeloid cells (MC) recruitment and microglia activation in brain. PGN or PBS was stereotaxically injected into the mouse caudate putamen (CPU). **(A)** At 72 h after injection, 10 µm brain sections were stained with an anti-CD45 (green) antibody. The slides were analyzed under a laser scanning confocal fluorescence microscope. In the PBS-quadrant, arrowheads indicate ramified resting parenchymal microglia, since in the PGN-quadrant, arrowheads indicate activated parenchymal microglial cells. **(B)** At indicated time points after injection, coronal brain slices of 2.0 mm containing the CPU from the injected hemisphere [caudate putamen (CPU)] were dissected. The tissue was homogenized, and CPU cells were costained with anti-CD45 (APC-Cγ7) and anti-CD11b (PERCPE) antibodies and analyzed by flow cytometry gating on $CD11b^{+}$ $CD45^{+}$ MC. The bar graph represents mean ± SD of number of MC from three separated experiments. **(C)** At 72 h after injections, coronal brain slices containing the CPU were photographed. Circle indicate injured injection site. These brain sections were stained with anti-CD45 (green) and then were analyzed under a laser scanning confocal fluorescence microscope, injection site is showed in the picture. **(D)** At 72 h after injection, CPU from injected hemisphere (Ipsilateral-CPU), or CPU of non-injected hemisphere (Contralateral-CPU), or the whole brain (BRAIN) were processed such as indicated in point B. Then, this brain cells were analyzed by flow cytometry gating on $CD11b^{+}CD45^{high}$ infiltrating myeloid cells (IMC) and gating on $CD11b^{+}CD45^{low}$ resident myeloid cells (RMC). **(E–G)** The bar graph represents mean ± SD of number of MC, of IMC, and of RMC from three separated experiments, respectively. * and *** indicate statistically significant ($p < 0.05$ and $p < 0.001$, respectively) changes compared with PBS-injected mice.

these inhibitors were observed at the whole brain (**Figures 3C,D**). Taking into consideration that LY294002 and 3-MA inhibited the response to PGN and these molecules can inhibit autophagy, these results suggest that PI3K and autophagy response may be required for the MC recruitment in PGN-injected brains.

TLR2 and MyD88 Signaling Regulates PGN-Induced Leukocyte Entry to the CNS Parenchyma

To confirm the involvement of TLR2 and MyD88 adaptor protein in the recruitment effects of PGN, we studied leukocyte recruitment to mouse brain parenchyma after PGN intracerebral injection in TLR2KO and MyD88KO mice. We found that recruitment of MC in parenchymal CNS was significantly lower in TLR2KO mice compared with the WT mice, 72 h after PGN injection (**Figures 4A,B**). Consistent with this result, we observed that PGN injection in TLR2KO mice failed to increase the number of IMC, RMC in the ipsilateral-CPU and in whole brain, compared with the PGN-injected WT mice (**Figures 4A,B**). Similar results were obtained in MyD88KO mice, since we did not observe the major MC recruitment in the ipsilateral-CPU and whole brain of MyD88KO mice after PGN injection (**Figures 4A,B**). Taken together, our experiments demonstrate that the activation of

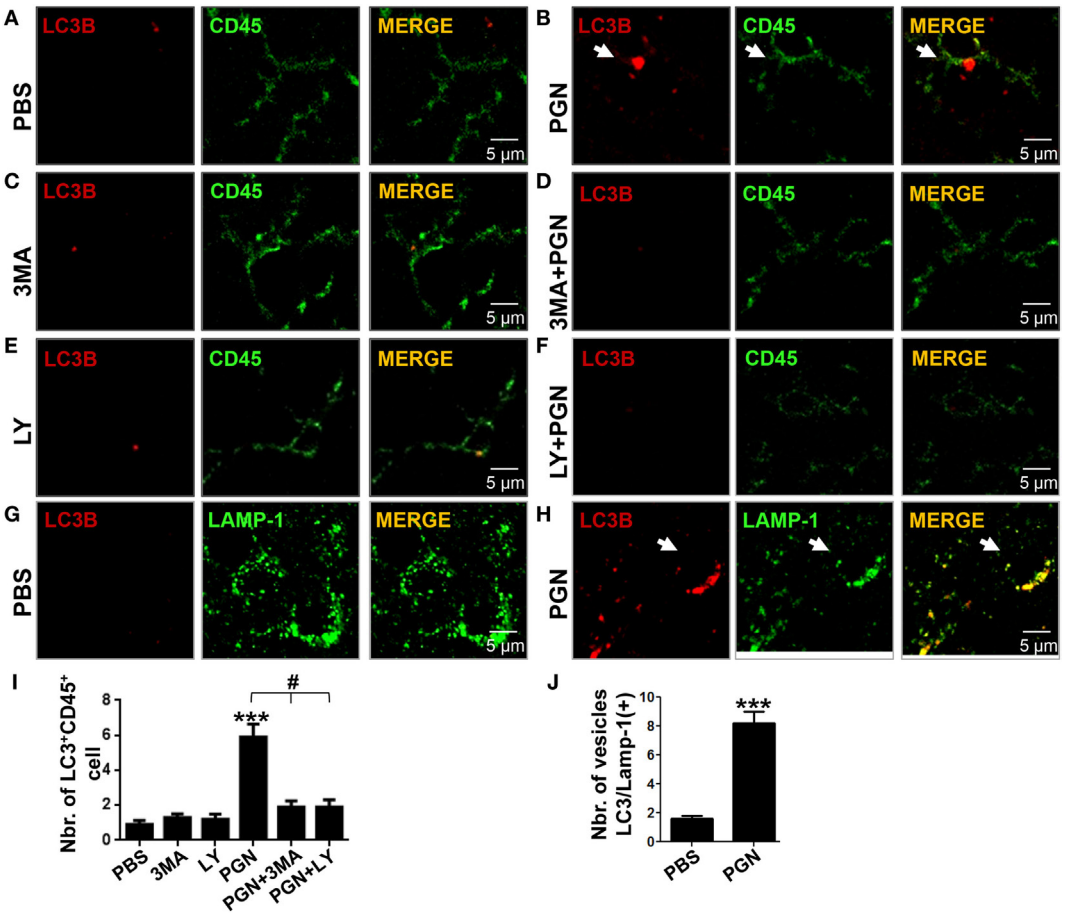

FIGURE 2 | PGN induces myeloid cell autophagy in brain inflammation model. PBS, 3MA, or LY2942002 was stereotaxically injected alone or in combination with PGN into the caudate putamen. **(A–F)** After 24 h, 10 µm brain sections were stained with anti-LC3B (red) plus anti-CD45 (green) or anti-LAMP-1 (green) antibodies. The slides were analyzed under a laser scanning confocal fluorescence microscope. **(A–F)** Arrows indicate the presence of LC3B positive punctuated cells. **(G,H)** Arrowheads indicate colocalization of LC3B positive vesicles with LAMP-1. **(I)** The bars graph represents mean ± SD of number of LC3B$^+$ vesicles per CD45$^+$ cell of three separated experiments. **(J)** The number of LC3B/Lamp-1 double-positive cells was obtained from 10 fields per slide, analyzing the next three sections without the needle artifact of three separated experiments. *** indicate statistically significant ($p < 0.001$) changes compared with un-stimulated cells.

TLR2 by PGN and signaling through the MyD88 adaptor protein are an important regulator of leukocyte recruitment after PGN injection in the brain parenchyma.

As showed above, PI3K inhibitors regulate MC entry to PGN-injected CPU. As expected, in additional experiments, we observed that coinjection of PGN plus 3-MA or PGN plus LY294002 in TLR2KO and MyD88KO mice, did not reveal significantly differences in the leukocyte recruitment in the ipsilateral-CPU and whole brain of these mice compared with the controls (**Figure 4C**). Thus, this experiment suggests that PI3K inhibitors does not affect leukocyte entry to CNS parenchyma, in the absence of TLR2/MyD88-mediated PGN signaling.

PI3K Inhibitors Modulate Inflammatory Cell Phenotype and Pro-Inflammatory Mediators in PGN-Injected CPU

Expression of MHC class II and CD80 and CD86 costimulatory molecules is associated with the ability to present antigen, and their detection can be used to indicate MC activation (48). Previous studies demonstrated that LPS (TLR4 ligand) was able to increase MHC class II, CD80, and CD86 expression in primary microglia isolated from human adult patients (49). However, there is little evidence in the literature if another TLR family members, such as TLR2, could regulate activation of microglia, within damaged CNS. Here, we explored the expression of molecular markers related to the cell activation, in MC from PGN-injected mice, in the presence/absence of autophagy inhibitors. We found that PGN injection increased expression of MHC class II and CD86 in microglial cells and monocytes. Interestingly, 3-MA and LY294002 treatment prevented the effects of PGN on the expression of both molecular markers in these cell types (**Figures 5A,B**). Chemokines and their receptors are expressed in the CNS, where they play key functions in development and maintenance (50, 51). For instance, CX3CL1/CX3CR1 signaling modulates stimulus-dependent microglial activation (52). Moreover, CXCR4 is upregulated in microglia and astrocytes in various brain diseases, such as HIV encephalitis

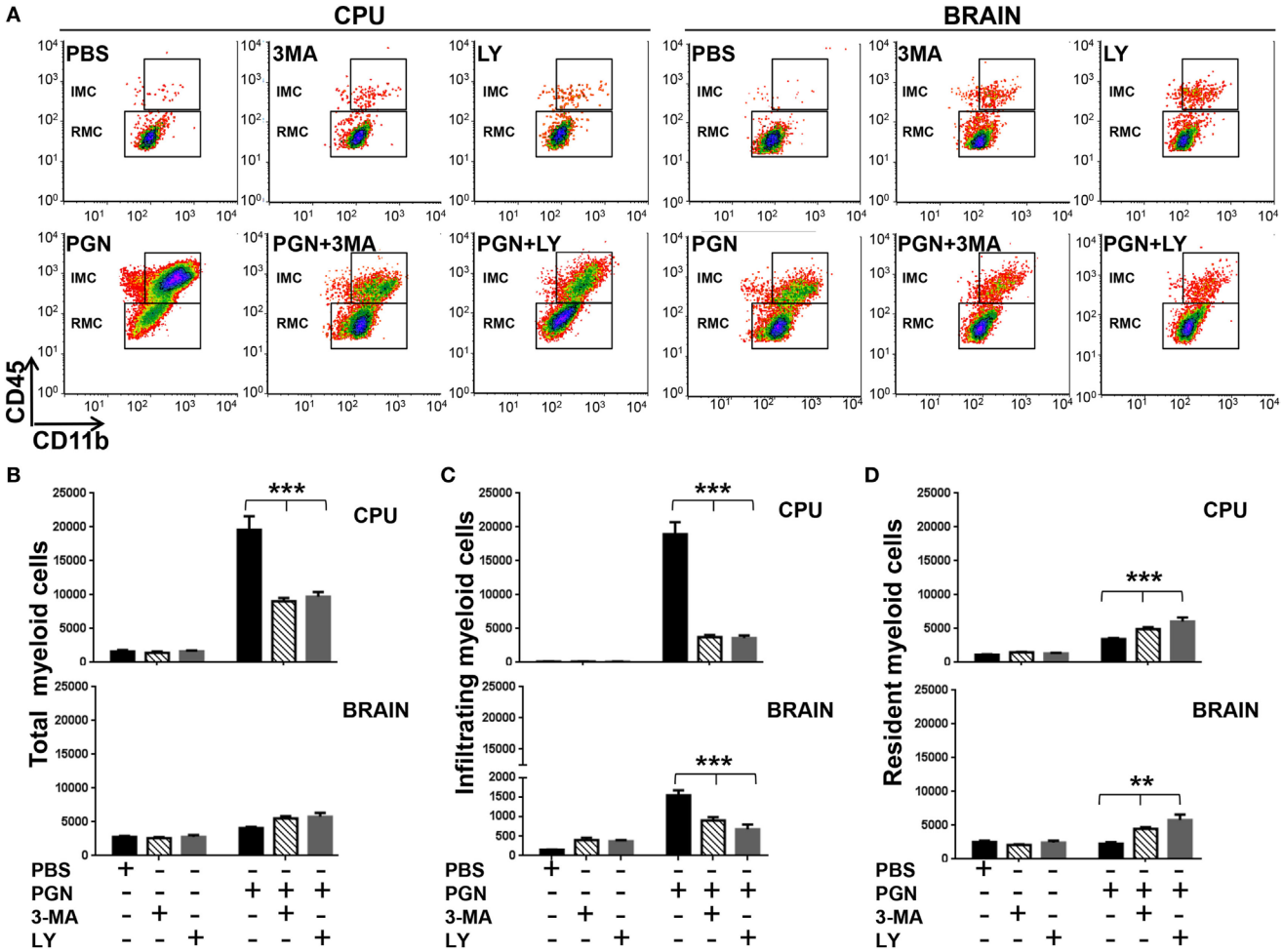

FIGURE 3 | PGN-induced myeloid cell recruitment requires PI3K. PGN or PBS was injected with 3-methyladenine (3-MA) or LY2942002 into the caudate putamen (CPU). **(A)** At 72 h after injection, CPU or BRAIN cells were analyzed by flow cytometry gating on MC. **(B–D)** The bar graph represents mean ± SD of number of MC, of infiltrating myeloid cells and of resident myeloid cells from three separated experiments. * and *** indicate statistically significant ($p < 0.05$ and $p < 0.001$, respectively) changes PGN + 3-MA or PGN + LY2942002 compared with PGN-injected mice.

and experimental allergic encephalomyelitis (53, 54). We investigated the expression of CXCR4 and CX3CR1 in microglia and infiltrating monocytes after PGN intracerebral injection. We observed that PGN increased CXCR4 and CX3CR1 expression in microglia compared to the control, and their expression was significantly inhibited in the presence of LY294002 (**Figure 5C**). However, this inhibitor did not affect the chemokines expression in monocytes from PGN-injected mice (data not shown). In addition, we investigated whether CCL2 and CCR2 are increased in PGN-injected mice compared with controls. This signaling axis is important on monocyte recruitment to CNS tissue during immune-mediated inflammation (55). We detected increased CCL2/CCR2 gene expression in PGN-injected CPU, compared with controls (**Figure 5D**), which was prevented by LY294002 injection (**Figure 5D**). Microglia activation is associated with the secretion of cytokines that later can modulate their activation state. High levels of pro-inflammatory cytokines such as TNF-α, IL-6, and IL-1β were found elevated in the brain tissue from patients with neurological diseases (56). It has also been reported that PGN increases iNOS and COX-2 expression in BV2 microglial cells by binding to the TLR2 receptor/MyD88 which in turn activates PI3K/AKT/NF-kappa B signaling pathway (57). Here, we observed that PGN injection promoted iNOS, IL-1β, IL-6, and TNF-α gene expression compared with controls (**Figure 5E**). In addition, we studied if PI3K inhibition modulates pro-inflammatory cytokines expression after PGN treatment. We found that coinjection LY294002 with PGN into CPU, prevented PGN-induced iNOS and IL-1β production (**Figure 5E**), but PGN-induced IL-6 production was increased in presence of this inhibitor (**Figure 5E**). Moreover, LY294002 was not able to change PGN-induced TNF-α production (**Figure 5E**). These results suggest that PI3K inhibitors could selectively regulate iNOS and IL-1β production, in addition to leukocyte recruitment, in PGN-injected mice.

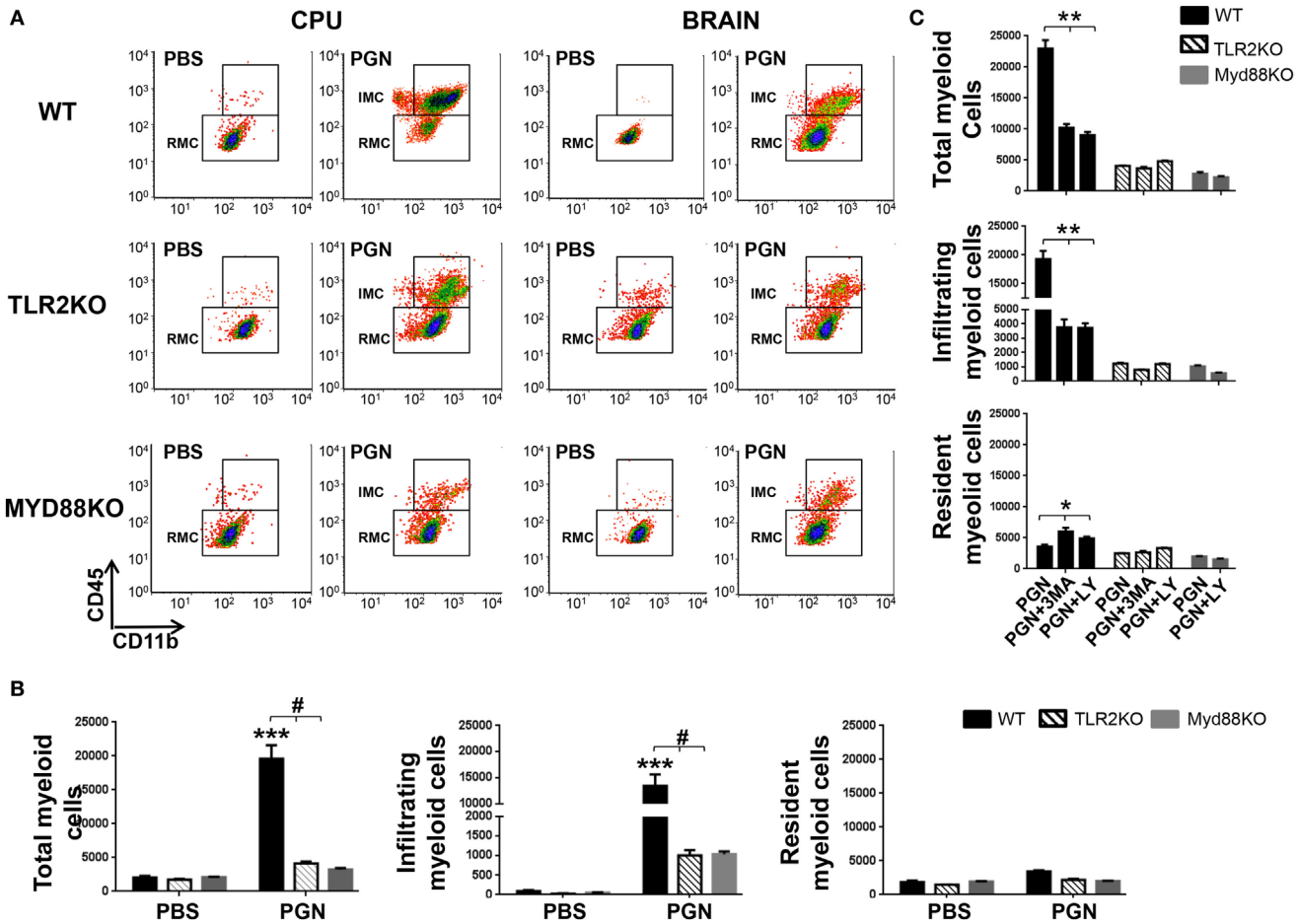

FIGURE 4 | PGN-induced myeloid cell recruitment requires MyD88/TLR2 signaling. **(A)** PGN or PBS was injected into the caudate putamen (CPU) from MyD88 KO, TLR2KO, or C57BL/6 mice. At 72 h after injection, CPU or BRAIN cells were analyzed by flow cytometry gating on MC. **(B)** The bar graph represents mean ± SD of number of MC, of infiltrating myeloid cells (IMC) and of resident myeloid cells (RMC) from three separated experiments. * and *** indicate statistically significant ($p < 0.05$ and $p < 0.001$, respectively) changes PGN-injected TLR2 KO mice or PGN-injected MyD88 KO mice compared with PGN-injected wild-type (WT) mice. **(C)** The bar graph represents mean ± SD of number of MC, of IMC, and of RMC from three separated experiments. * and *** indicate statistically significant ($p < 0.05$ and $p < 0.001$, respectively) changes PGN + 3-methyladenine (3-MA) or PGN + LY2942002 injected WT mice compared with PGN-injected WT mice.

PI3K Inhibitors Prevent PGN-Induced Neuronal Cell Death

Finally, we examined if PGN was able to induce neuronal damage in mouse brain, using FJB staining and confocal microscopy. We detected neuronal toxicity generation in CPU sections of PGN-injected mice compared with the controls (**Figures 6A,C**). Furthermore, we observed that the coinjection of LY294002 reduced neuronal cell death induced by PGN (**Figures 6B–D**). Therefore, these data suggest that PI3K signaling regulate neuronal damage induced by PGN injection.

DISCUSSION

Recent studies have provided key insights into TLR-mediated activation of PI3K, suggesting that the outcome of PI3K activation downstream of these immune receptors depends on both the TLR that is stimulated and the cell type being activated (34). In this study, we have uncovered a completely novel role for PI3K in the control of brain inflammation. We have shown that the PGN injection into the brain parenchyma triggered LC3B$^+$ puncta in CD45$^+$ cells, LC3B and LAMP1 colocalization, recruitment of MC to the injection site, pro-inflammatory mediators' production, and neurodegeneration. Moreover, these effects of PGN were prevented by PI3K inhibitors (including 3-MA). To the best of our knowledge, this is the first demonstration that class III and class I PI3K inhibitors, that may also modulate autophagy, a fundamental homeostatic cell process, could control inflammatory response in the CNS after TLR2 stimulation.

Myeloid cells in CNS represent a heterogeneous class of innate immune cells that contribute to the maintenance of tissue homeostasis differentially during development and adulthood (58, 59). Microglia are the only MC type in the CNS parenchyma under the steady-state conditions and act as the first line of

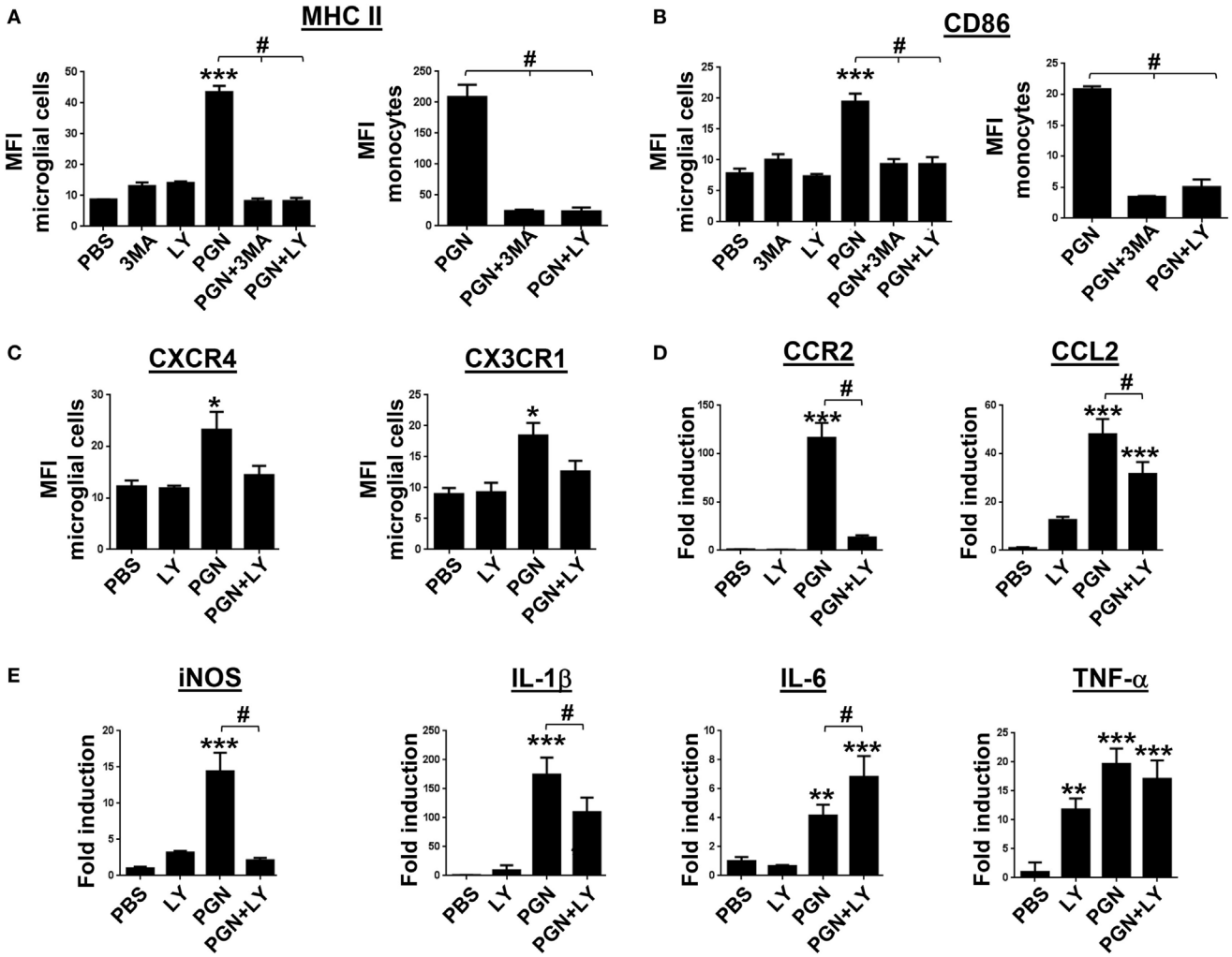

FIGURE 5 | Inhibition of PI3K modulates PGN-induced pro-inflammatory myeloid cells activation. PGN or PBS was injected with 3-methyladenine (3-MA) or LY2942002 into the caudate putamen (CPU). **(A–C)** At 72 h after injection, CPU cells were stained with anti-IA/IE, anti-CD86, anti CXCR4, and anti-CX3CR1 antibodies and analyzed by flow cytometry gating on microglial cells ($CD11b^{+}CD45^{low}$ cells) or monocytes ($CD11b^{+}CD45^{high}Ly6C^{+}$). The bar graph represents mean ± SD of mean fluorescence intensity (MFI) from three separated experiments. * and *** indicate statistically significant ($p < 0.05$ and $p < 0.001$, respectively) changes PGN compared with PBS-injected mice, # indicate statistically significant ($p < 0.05$ and $p < 0.001$, respectively) changes PGN + 3MA or PGN + LY2942002 compared with PGN-injected mice. **(D,E)** At 72 h after intracerebral injection, total RNA was extracted from CPU tissues and examined for mCCR2, mCCL2 miNOS, mIL-1β, mIL-6, and mTNF-α genes expression by real-time PCR. Arbitrary units were used to indicate the fold difference in PGN versus PBS-injected mice after normalization with the HPRT transcripts. # indicate the fold difference in PGN + LY2942002 versus PGN-injected mice.

defense in the nervous system (8). The morphologically "resting" microglia are continually surveying their microenvironment with their highly motile processes. Upon detection of focal injury to the brain, microglial processes rapidly move toward the lesion site, and switching their behavior, leading to focal microglial activation (35). This work provides evidence that PGN-induced focal activation of microglia confined to the CPU. We observed microglial cell accumulation with morphology distinguishable from the resident microglial cells, consisting of shorter, asymmetrically oriented processes as well as enlarged cell bodies nearly to the injection site. In addition, we demonstrated that PGN present in the mouse brains not only induced microgliosis but also augmented other CNS-infiltrating MC populations in the injected site. In line with these findings, other authors previously reported that PGN in the brain of multiple sclerosis patients could contribute to the disease progression through the activation of infiltrating dendritic cells (60). Recently, Luz et al. found that TLR2 activated in CNS affects the innate but not adaptive brain immune responses. Our findings revealed that stimulation of TLR2 in brain parenchyma (CPU) triggers MC recruitment in the several areas analyzed (61). We observed that PGN increased MC numbers in CNS 72 h after injection, but this response was controlled 14 days after treatment. This evidence suggests that the parenchymal PGN injection could temporally activate CNS MC, which mediate local inflammation and later these cell populations return to steady state.

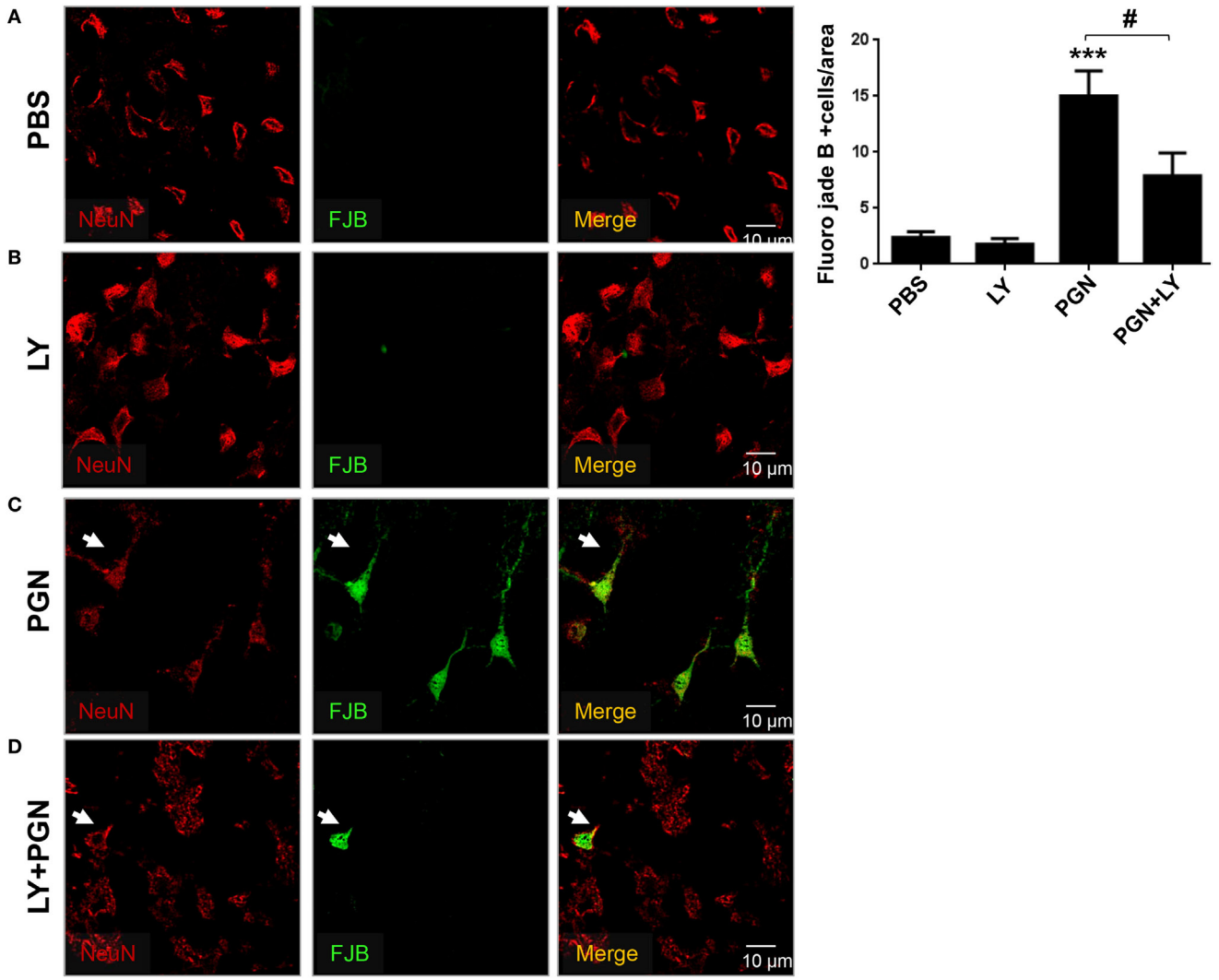

FIGURE 6 | Pharmacological inhibition of PI3K prevents neuronal loss in PGN-injected brain. PGN or PBS was injected with LY2942002 into the caudate putamen. **(A–D)** At 72 h after injection, 20 µm brain sections were stained with anti-NeuN (red) antibody. Then, the slides were stained with Fluor-Jade B (FJB) work solution and analyzed under a laser scanning confocal fluorescence microscope. Arrowheads indicate the presence of FJB positive neuron cells. The bars graph represents mean ± SD of number of FJB + neuron cells from three separated experiments. *** indicate statistically significant ($p < 0.001$) changes PGN compared with PBS-injected mice.

Under normal conditions, microglial cells contribute to the restriction of other MC to access the CNS parenchyma. However, neuroinflammatory circumstances, such as neural injury or local inflammation, trigger defined molecular mechanisms that allow other non-parenchymal mononuclear phagocytes (those found in the choroid plexus, meninges, and perivascular spaces) and peripheral immune cells to breach the glia limitans and enter the CNS parenchyma (62, 63). We observed that PGN injection into brain parenchyma lead to the migration of MC, which were mostly confined around the injection site. Interestingly, two PI3K inhibitors, such as 3-MA and LY294002, were able to block this MC recruitment. Taking into consideration that 3-MA prevent autophagosome formation, these results indicate that microgliosis and recruitment of others MC induced by TLR2 activation may require autophagy activation. These results are in agreement with a recent publication showing that genetic inhibition of autophagy prevented irradiation-induced microglial activation and neuroinflammation (64). On the other hand, Jin et al. found that intracerebral administration of the autophagy inhibitor 3-MA promoted microglia activation after traumatic brain injury (65). Taking into consideration the results of the present study and other reports, we propose that autophagy may play different roles in modulation of MC activation and inflammation, depending on the inflammatory context and other microenvironment factors. For instance, basal levels of autophagy are required for normal neuron survival (28, 66), while overactivated autophagy induces autophagic cell death (67).

Although no solid evidence about the role of MC autophagy on brain inflammation has emerged, recent research showed that neuronal TLR2 activation has been associated with the increased levels of the autophagy/lysosomal pathway marker p62 as well as with the recruitment and activation of microglia in the substantia nigra of PD brain (68). In this sense, autophagy is regulated by a plethora of immunological signals, including ligands for pattern-recognition receptors, for example, TLRs and cytokines (69, 70). In this article, we showed that PGN, a TLR2 ligand, triggers autophagy activation in brain MC and autophagolysosome formation, and this effect was blocked by PI3K inhibitors. Considering all these results, we explored the role of TLR2 signaling in PGN-induced brain inflammation. We observed that TLR2 and the common TLR signaling adaptor MyD88 were required for the PGN-induced MC recruitment and MyD88 and TLR2 deficiency mainly affected infiltrating MC accumulation instead resident MC. In accordance with our results, it was described in the literature that MyD88/TLR2 signaling regulated infiltration of the peripheral immune cells populations and microglial expansion in response to the acute brain inflammation (71, 72). Thus, we confirm that MyD88/TLR2 signaling plays a pivotal role in PGN-induced inflammatory response and this effect may involve PI3K and autophagy activation in CNS MC.

Several pro-inflammatory factors produced by CNS MC also influence the evolution of neuroinflammatory injury (73). Previous studies have shown that inflammation leads to the accumulation of perivascular macrophages and microglial cells at the inflammatory site, and they upregulate MHC class II expression and costimulatory molecules (74). In agreement with these studies, we also revealed that PGN injection increased MHC-II and CD86 expression in microglia and monocytes at the inflammatory site and PI3K inhibition reduced these PGN effects. These findings are in agreement with the concept that MC in the CNS are highly specialized but also plastic cells, that become reactive in the context of any changes in CNS homeostasis. In this sense, we suggest that the PI3K pathway participate in determining the phenotypic profile of MC and its inhibition could lead to limitation of CNS inflammation.

Multiple mechanisms could account for the induction in MC recruitment observed in PGN-injected mice. Based on previous reports describing that fractalkine/CX3CR1 signaling regulates microglial behavior in several CNS disorders (75), we evaluated the CX3CR1 expression in microglial cells after PGN treatment. Interestingly, since we observed that PGN increased CX3CR1 expression in these cells, LY294200 was able to attenuate this PGN-effect. In addition, we found that PGN also increased CXCR4 expression in microglial cells, and this upregulation was diminished by PI3K inhibition. These findings suggest that inhibition of PI3K could regulate inflammatory response in CNS from PGN-injected mice involving mechanisms that reduce migration of peripheral MC to injury site and modulate phenotype profile of resident MC.

In pathological conditions, microglia can activate endothelial cells by the release of reactive oxygen species and cytokines such as IL-1α, IL-1β, IL-6, and TNF-α (76, 77). Also, the recruitment of peripheral immune cells can be initiated by microglia through the secretion of the chemokine CCL2 under inflammatory conditions (78). Here, we demonstrated that PGN promoted and increase in iNOS, IL-1β, IL-6, and TNF-α gene expression in cells from the injection site. Moreover, PI3K inhibition differentially modulated gene expression of these mediators. Finally, in this set of experiments, we observed an increase in CCR2 and CCL2 gene expression in CPU from the PGN-injected mice compared with the control animals. Interestingly, LY294200 diminished gene expression of both molecules studied.

There are different glial cell populations (astrocytes and microglial cells) and infiltrating MC, responsible for the release of inflammatory mediators in the CPU from PGN-injected mice. Therefore, we suggest that the global effect of PI3K inhibition of inflammatory mediator expression may be affected by the presence of different cell types, that may produce distinct mediators, since PI3K signaling modify cell recruitment.

Brain inflammation is a typical feature of neurodegenerative diseases (79–81) and could be prominent sequel of many acute forms of brain injury (for example, trauma, encephalitis, and stroke) (82, 83). Under certain circumstances, neuroinflammation is known to promote neuronal death (84). Our findings suggest that PGN induced neuronal cell death involving autophagy activation. Indeed, our studies have shown that MC exposed to PGN failed to be toxic to neighboring neurons in the presence of LY294200.

In this sense, was reported that autophagy activation in cerebral ischemia has a destructive role but this effect was prevented by the administration of 3-MA (85, 86). Conversely, other evidences have suggested that autophagy has a neuroprotective function (87, 88). We support the idea that physiological levels of autophagy are favorable to neuronal survival, but excessive or inadequate levels, could be harmful and cause injury.

To summarize, this study suggests a new role for PI3Ks and PGN-induced autophagy, in MC recruitment to the CNS and brain inflammation. Considering that recent discoveries point to autophagy as a substantial regulator of CNS innate immune responses and our findings that pharmacological inhibitors of class I and class III PI3K can suppress both, the inflammatory response and neuronal toxicity, we proposed that these molecules could potentially be consider as targets for exploration and development of new therapeutic strategies for neurodegenerative diseases.

ETHICS STATEMENT

All experiments were done in compliance with the procedures outlined in the "Guide for the Care and Use of Laboratory Animals" (NIH Publication No. 86-23, 1985). The experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC). Our animal facility obtained NIH animal welfare assurance (No. A5802-01, OLAW, NIH, USA).

AUTHOR CONTRIBUTIONS

DA designed and carried out all the experiments and wrote the manuscript. EG helped in setting up *in vivo* experiments

and confocal microscopy studies. JR and CB helped to perform *in vivo* studies. MA and LC helped in setting up and carried out intracerebral injections. PI conceived and designed the study, supervised all of the experiments, and helped to write the manuscript. All of the authors discussed the results and commented on the manuscript.

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REVIEW
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Immune Microenvironment in Glioblastoma Subtypes

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Glioblastomas (GBMs) are the most common and aggressive primary brain tumors. Due to their malignant growth and invasion into the brain parenchyma coupled with resistance to therapy, GBMs are among the deadliest of all cancers. GBMs are highly heterogeneous at both the molecular and histological levels. Hallmark histological structures include pseudopalisading necrosis and microvascular proliferation. In addition to high levels of intratumoral heterogeneity, GBMs also exhibit high levels of inter-tumoral heterogeneity. The major non-neoplastic cell population in the GBM microenvironment includes cells of the innate immune system called tumor-associated macrophages (TAMs). Correlative data from the literature suggest that molecularly distinct GBM subtypes exhibit differences in their microenvironment. Data from mouse models of GBM suggest that genetic driver mutations can create unique microenvironments. Here, we review the origin, features, and functions of TAMs in distinct GBM subtypes. We also discuss their interactions with other immune cell constituents and discuss prospects of therapeutically targeting TAMs to increase the efficacy of T-cell functions.

Keywords: glioblastoma, macrophages, microglia, immunotherapy, tumor-associated macrophages, tumor microenvironment

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INTRODUCTION

Glioblastomas (GBMs) are the most common and aggressive malignant primary brain tumors in adults (1). GBM cells are characterized by diffuse infiltration of the adjacent brain parenchyma and the development of resistance to standard treatment (2–4). The standard of care consists of surgical resection followed by radiotherapy (RT) and concomitant and adjuvant temozolomide [(TMZ); TMZ/RT→TMZ]. Despite this aggressive treatment regimen, the median survival is only around 15 months, and the 2-year survival rate is only 26.5% (5).

Glioblastomas were traditionally considered to be a single histological entity by the World Health Organization. However, a more recent characterization of the genome, epigenome, and transcriptome of GBMs has provided a higher-resolution picture of frequent alterations, based on which robust gene expression-based subtypes named proneural (PN), mesenchymal (MES), and classical (CL) were established (6–10). These analyses associated aberrations in the gene expression of platelet-derived growth factor receptor alpha (PDGFRA), neurofibromatosis type I (NF1), and epidermal growth factor receptor (EGFR) with the PN, MES, and CL subtypes, respectively. Although it is important to emphasize that multiple subtypes could co-exist within a single tumor both at the regional and at the single-cell levels (11, 12), the designated subtypes reflect the dominant transcriptional program of a specific tumor within a particular time and space of sample isolation (10). By analyzing copy number alterations from The Cancer Genome Atlas (TCGA) data to evaluate the presence of NF1 loss, PDGFRA amplification, and EGFR amplification in human GBM (hGBM) samples when co-incidence of mutations was excluded, we demonstrated that NF1 loss, PDGFRA amplification, and

EGFR amplification tend to occur most frequently in MES, PN, and CL hGBMs, respectively (13, 14).

As described above, GBMs display a high degree of inter- and intratumor heterogeneity. The tumor microenvironment, in which these tumor cells develop and grow, further adds to this diversity. The GBM microenvironment contains an array of non-neoplastic cells, including infiltrating and resident immune cells, vascular cells, and other glial cells. Particular emphasis has been placed on various non-neoplastic constituents of the immune system, especially tumor-associated macrophages (TAMs). TAMs are the dominant infiltrating immune cell population, constituting ~30–40% of the cells in a GBM (15, 16). These cells have been shown to engage in reciprocal interactions with neoplastic tumor cells to promote tumor growth and progression (17–20). With the advent of immunotherapeutic strategies for GBM, T cells have also been the subject of increasing scrutiny (21, 22). These innate and adaptive immune cells together form the basis of our host defense, where they perform cancer immune surveillance at early stages of premalignant lesions. However, if and when the immune system is overpowered by tumor burden during cancer development, cancers can escape this surveillance and become uncontrollable. In doing so, cancers also recruit these immune cells and methodically turn them into their accomplices (23), effectively converting the immune system from protective to detrimental. The task we are facing now as immuno-neuro-oncologists is to re-educate and re-invigorate these immune cells and to rectify their actions to be once again advantageous. This review aims to analyze the most recent findings and to assess whether genetic driver mutations can determine the expression profile of non-neoplastic cells and/or can play an important role in predicting tumor response to immunotherapy. Our goal is to promote discussion with regard to subtype-oriented immunotherapies and to advocate for such considerations.

IMMUNE COMPOSITION OF GBM SUBTYPES

Since the contribution of TAMs to tumor development is substantial, several studies utilizing gene expression data from the TCGA and the Gene Expression Omnibus databases have demonstrated an enrichment in immune response-related gene expression, especially of TAM genes, in the MES subtype of GBM compared to the other subtypes (15), suggesting that TAMs could play a subtype-specific role in GBM. Despite extensive correlative studies and *in vitro* experiments implying that TAMs may play differential roles in GBM subtypes, to date, there are still no systemic functional studies corroborating this hypothesis. On the contrary, despite emerging evidence from both mouse models and TCGA analysis of hGBM (10) showing that NF1 deficiency results in an increased TAM infiltration, the clinical significance of this finding is not apparent. Clinically, the subtypes have not been established as predictive biomarkers for survival (8), although accumulating preclinical evidence has indicated that subtype-specific treatment may preferentially benefit patients. It is still not understood, however, what controls the differences in immune composition in GBM subtypes. One scenario is that tumor-associated or tumor-specific antigens, driven by genetic mutations, are differentially presented in different subtypes, which shapes the various molecular immune responses and results in the observed differential accumulation of immune cells (8, 24).

Glioblastoma creates a proangiogenic and inflamed microenvironment, which leads to an increased expression of adhesion molecules on the endothelial cells and reduced tight junctions, thereby a highly permeable blood–brain barrier (BBB). These changes support the leukocytes to exit from the blood flow by extravasating through the brain endothelial wall and infiltrate the tumor mass. Besides TAMs, many other immune cells are also found in the GBM parenchyma, although at a much lower incidence. T cells probably account for most of the lymphoid cells in GBMs; however, they represent less than 0.25% of total tumor cells isolated from hGBM biopsy samples as examined by flow cytometry (25). $CD8^+$ cytotoxic T cells are cellular immune effectors that are essential for killing tumor cells, but they are only sparsely distributed in the GBM parenchyma, accounting for less than a quarter of the total $CD3^+$ T cells (25). These T cells derived from GBM patients are less responsive to direct anti-CD3 stimulation *in vitro* when compared to cells obtained from healthy controls, indicating an immunosuppressed status (25). In support of this notion, it was recently shown that GBM-infiltrating T cells increased their expression of indoleamine 2,3-dioxygenase 1 (IDO1), which is an immune-inhibitory receptor and that this heightened expression correlates with poor prognosis (26). A phase I clinical trial examining the safety and utility of an IDO1 inhibitor in conjunction with TMZ in pediatric primary malignant brain tumors is currently underway (clinicaltrials.gov NCT02502708). Regulatory T cells (T_{regs}) are also found in the GBM parenchyma. These cells perform immunosuppressive functions and are thought to suppress antitumor immunity in various solid tumors such as ovarian, breast, and pancreatic cancers (27). In GBM tumor cells, secreted soluble factors including CCL22 can facilitate the recruitment and retention of T_{regs} in the tumor microenvironment (28), and the amount of T_{regs} demonstrated an inverse correlation with patient survival, although it was not statistically significant (29). T_{reg} ablation eradicates T-cell-proliferative defects, restoring the functions of T cells from GBM patients *in vitro* at levels equivalent to those of healthy controls (30). Therefore, targeting T_{regs} can potentially revert tumor immune evasion, thereby facilitating tumor immunotherapy or conventional therapy.

In silico estimation of 22 immune cell types in human PN, CL, and MES samples has shown that there is a collective increase in several cell types in MES tumors compared to that in non-MES tumors, including $CD4^+$ memory T cells, type-2 polarized macrophages, and neutrophils (10). It has been speculated that a higher level of TAMs may discourage the infiltration of effector T cells due to TAM immunosuppressive functions. However, the reasons for this hand-in-hand infiltration between TAMs and T cells in a subtype-specific manner are not evident. This could be because the T cells follow the TAMs to passively egress the bloodstream when the BBB is compromised during GBM development. However, this is unlikely in that the ratio of T cells to TAMs in the tumor is different than that in the blood, where lymphocytes considerably outnumber monocytes (progenitors of

tumor TAMs). One possible explanation could be that there is a parallel increase in CCL chemokines (attracting monocyte) and CXCL chemokines (attracting lymphoid cells) in MES tumors that attract TAMs and T cells, respectively, when compared with other GBM subtypes.

Genetically engineered mouse models (GEMMs) that faithfully recapitulate hGBM subtypes are invaluable tools for enabling the investigation of subtype-specific immunopathology and for the design of relevant and effective therapies (14, 31). These specific GEMMs provide an unprecedented opportunity to define the immune cells and molecular signals that contribute to gliomagenesis and continued growth facilitated by subtype-specific glioma microenvironments. For specific questions regarding tumor–microenvironment interactions, GEMMs for various GBM subtypes represent better choice compared to other models, such as orthotopic murine allografts utilizing established murine GBM cell lines, cultured in serum for years, or hGBM xenografts, where there are well-known species incompatibilities, particularly for chemokines and their receptors. Among all of the desirable properties of these models is that they utilize immunocompetent mice, in which the immune cells and tumor cells are of the same species, eliminating species incompatibilities between chemokines, cytokines, and their respective receptors that are important for the recruitment and also the activation of various immune cell types. GEMM models of GBM will allow us to answer important biological questions regarding the relevance of differential immune infiltration in various hGBM subtypes.

TAMs: THE ORIGIN MATTERS

Tumor-associated macrophages originate from two independent sources: brain-resident microglia and/or bone marrow-derived monocytes (**Figure 1A**). Microglia is the unique resident macrophages of the central nervous system (CNS) (32). Fate-mapping and lineage-tracing studies have identified immature yolk sac runt-related transcription factor 1 (Runx1)-positive progenitors as the predominant source of brain microglia. Between embryonic days 8.5 (E8.5) and E9.5, these progenitors migrate from the yolk sac into the primitive brain, where they serve as cells of origin for microglia (33). Several additional studies have subsequently revealed in mice that myeloid progenitors from the blood do not significantly contribute to the pool of adult microglia after birth. Thus, the majority of adult microglia are yolk sac-derived and are maintained by virtue of their longevity and limited self-renewal (33–36). Tracing the life span of microglia by long-term *in vivo* single-cell imaging in mice, it has been shown that neocortical resident microglia can live for about 15 months on average, almost rivaling the life span of post-mitotic neurons (37). While the naïve CNS parenchyma is occupied exclusively by resident microglia, the tumor-bearing CNS is vastly different. In the tumor-bearing brain, the BBB is impaired, and the expression of the monocyte chemoattractant family of proteins (MCPs) is increased. This results in infiltration of monocytes into tumors from the periphery, where they differentiate into macrophages. Monocytes are derived from progeny called macrophage–DC precursors, which originate from hematopoietic stem cells. These precursors differentiate into monocytes within the bone marrow and are subsequently released into the blood circulation to colonize peripheral organs (38). Mouse monocytes can be further subdivided into two main populations: Ly6C$^+$, CX3CR1^{int}, and CCR2$^+$ inflammatory monocytes; and Ly6C$^-$, CX3CR1^{hi}, and CCR2$^-$ circulating monocytes (39, 40). It is well established that the Ly6C$^+$, CX3CR1^{int}, and CCR2$^+$ inflammatory monocytes leave the blood circulation and extravasate to inflamed tissues. Once homing to inflamed tissues, these cells gradually downregulate their CCR2 while concomitantly upregulating CX3CR1 as they differentiate into macrophages (41). Interestingly, TAMs exhibit a broad range of CX3CR1 and CCR2 expression levels in a reciprocal pattern

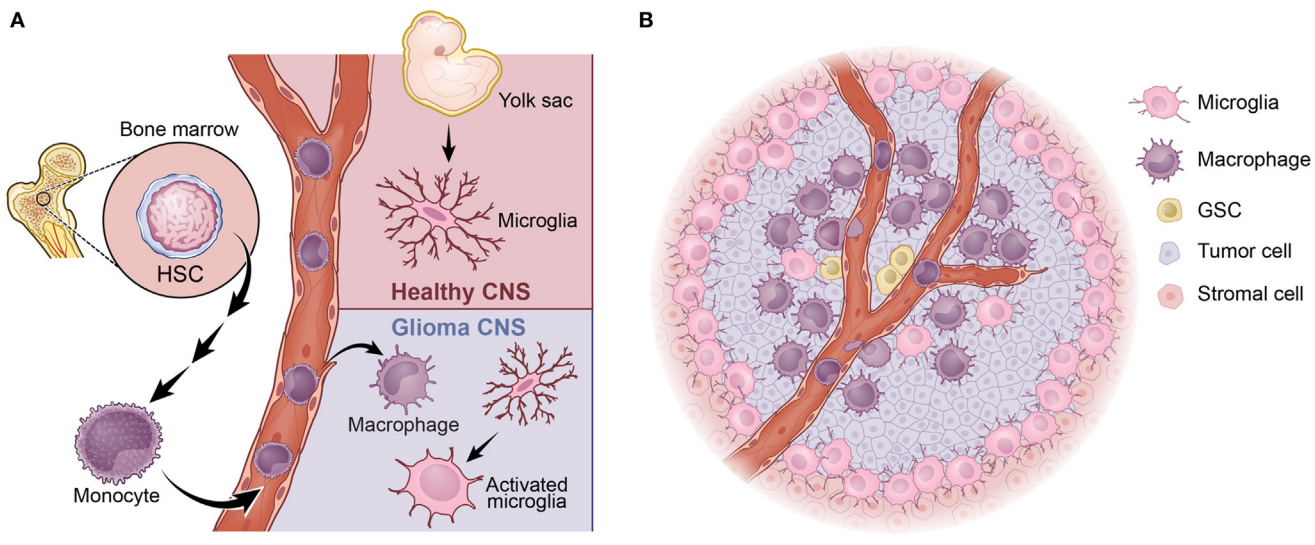

FIGURE 1 | Tumor-associated macrophages (TAMs) in glioblastoma (GBM). **(A)** TAMs arise from two distinct sources: bone marrow-derived monocytes or brain-resident microglia. **(B)** In proneural GBM, the majority of TAMs are BMDMs, which largely localize in the perivascular niche, where the glioma stem-like cells (GSCs) reside. The majority of microglia are found at the peritumoral region.

(i.e., decreasing CCR2 and increasing CX3CR1), indicating a continuous transformation of these cells from infiltrating monocytes into mature macrophages (42). This dynamic transition of the surface molecules suggests that bone marrow-derived monocytes are highly plastic and that these cells evolve to maturation *in situ* following localization to the tumors (43).

It has been established that bone marrow-derived macrophages and microglia react differently to various types of CNS insults and can perform different functions (44, 45). One example of this from a recent study using a complex parabiosis model showed that peripheral mononuclear cells invade the inflamed CNS during experimental autoimmune encephalomyelitis and play a significant role in disease progression to paralysis (46). By employing GEMMs of PDGFB-driven GBM described above, we have recently shown that the vast majority (up to 85%) of TAMs are infiltrating bone marrow-derived monocytes/macrophages, whereas resident microglia account for the remaining ~15% (42). Bone marrow-derived cells are prominent in perivascular areas, whereas resident microglia is more highly expressed in peritumoral regions (**Figure 1B**). RNA-sequencing analyses reveal that functional distinctions between bone marrow-derived and microglia-derived TAMs in that genes related to "cellular migration" are mostly enriched in the former, whereas genes associated with "pro-inflammatory cytokines" and "metabolism" are upregulated in the latter (42). These differences may be partially explained by the fact that these two cellular populations arise from distinct progenitors and selectively use different transcription factors for their gene regulation (47). To further illustrate their functional differences, we genetically deleted *Cx3cr1* from the microenvironment of PDGFB glioma-bearing mice and observed an increase in tumor incidence and a shortened survival time of stroma deficient in *Cx3cr1* compared to that in *Cx3cr1* wild-type stroma. These results showed that loss of *Cx3cr1* indirectly promoted trafficking of inflammatory monocytes into the CNS, resulting in a higher accumulation in the perivascular area (17). It did not, however, directly affect the accumulation of microglia in peritumoral regions. The bone marrow-derived monocytes promoted glioma stem-like cells by enhancing their proliferation through the production of IL-1β (17). These data strongly suggest that TAMs derived from the bone marrow compartment drive gliomagenesis, whereas microglia appears to play a less significant role in tumor growth and is mostly involved in tumor cell invasion. Together, these observations lead to several outstanding questions: (a) both human and mouse MES GBM exhibit an increased TAM infiltration when compared to the PN subtype, but do they exhibit different TAM compositions? (b) Is the number of TAMs or their composition more critical in promoting tumor development? (c) How different are TAMs in the CL subtype? Further, does the origin of a TAM matter for its interactions with T cells? These are very important questions that will provide novel insights, which can be used in designing successful immunotherapies aiming at killing tumor cells.

IMMUNOSUPPRESSION IN GBM

Tumor-associated macrophages are often considered to be facilitators of tumor growth because of their proangiogenic and immunosuppressive properties. Among these cells are those termed myeloid-derived suppressor cells (MDSCs). Broadly defined, MDSCs in mice are cells that express both CD11b and Gr1 surface markers, and they can be further subdivided into monocytic and granulocytic subtypes. In GBM, the granulocytic MDSCs are rarely found in the tumor (42). The monocytic MDSCs can employ a wide range of mechanisms to suppress cellular immune functions, including upregulation of Arg1 production, induction of T-cell apoptosis, and/or enhancement in the expansion of T_{reg} populations (48). All of these features align with the so-called M2 phenotype. *In vitro* studies initially demonstrated the dichotomous differentiation of macrophages, such that myeloid monocytes can be polarized into classically activated, pro-inflammatory (M1) or alternatively activated, anti-inflammatory (M2) phenotypes (49, 50). M1 cells produce high levels of oxidative metabolites and pro-inflammatory cytokines that are essential for host defense, but can also result in healthy tissue damage (51). On the other hand, M2 cells promote wound healing and suppress adverse immune responses (52). However, despite these initial findings in cell culture experiments, absolute M1 and M2 binary distributions are rare *in vivo*. Subsequently, a range of differentiation has been proposed, with the M1 and M2 phenotypes being at the ends of the spectrum (53). Indeed, in our transcriptome analyses of purified tumor-associated microglia and bone marrow-derived macrophages, we found mixed populations of both M1 and M2 phenotypes in both TAM populations (**Figure 2**). For instance, the typical M2 marker Arginase1 was upregulated by 10-folds (at $\log_2$ scale) in both bone marrow-derived macrophages and microglia, whereas IL-1β, a specific M1 cytokine, was also increased by 5-folds in both cell types. However, it is not immediately clear whether these M1 and M2 molecular signatures belong to distinct populations, or if a single cell can express both subsets of molecules at various strengths. What is clear is that TAMs are highly plastic and have been found to switch between M1 and M2 phenotypes in response to their environmental cues (54). Many attempts have been made to polarize TAMs to the M1 fate; however, sustained conversion remains a significant challenge because soluble factors produced by the tumor cells can revert TAMs to an M2 phenotype, despite pharmacological or genetic interventions. A comprehensive understanding of the molecular network that coordinates this conversion will benefit future attempts to maintain a long-lasting antitumor phenotype (55).

INHIBITION OR MODULATION OF TAMs AS A STROMA-DIRECTED STRATEGY

Given that TAMs are elemental accomplices in tumor development, it is reasonable to propose therapeutic options based on inhibiting their infiltration or promoting their demise. MCPs play an essential role in mediating monocyte migration and tissue infiltration. There are four MCP family members in humans—CCL2, CCL7, CCL8, and CCL13, whereas mice express CCL2, CCL7, CCL8, and CCL12. In the setting of murine GBM, we have shown that neoplastic cells in GBM express high levels of CCL2, which contributes to the directional infiltration of $CCR2^{Hi}$ inflammatory monocytes into the tumor (17). When we queried

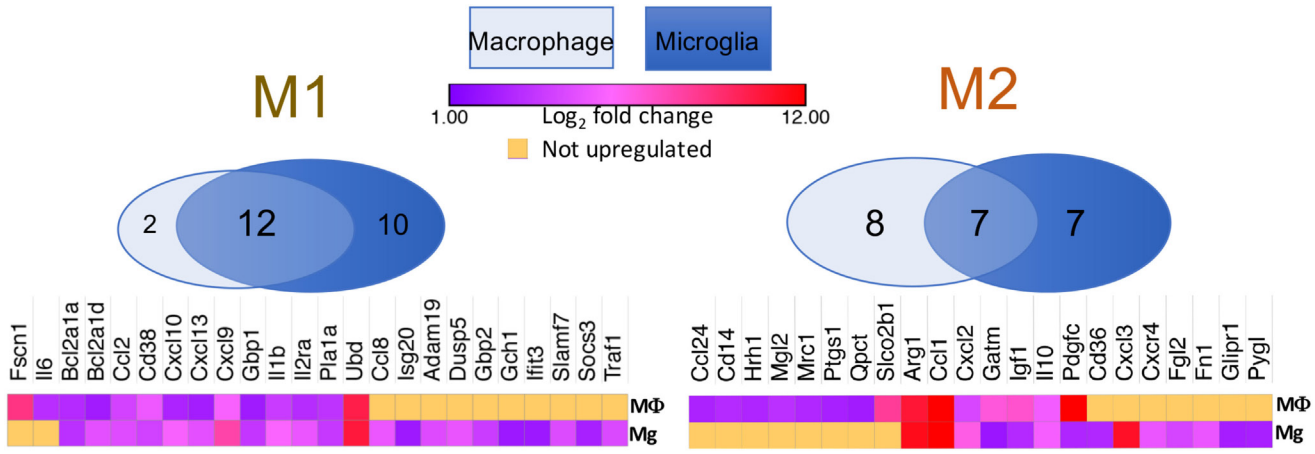

FIGURE 2 | Venn diagrams showing that both M1 and M2 signature genes are present in either BMDM or tumor-associated microglia isolated from a murine model of proneural glioblastoma. Heat maps demonstrate log_2-fold increases in these genes in BMDM and microglia as compared to their respective naïve controls. Raw RNA-seq data are available at the NCBI Sequence Read Archive database under accession number PRJNA349180 (42).

the human TCGA database for CCL2 expression and divided the patients into high and low CCL2 cohorts, we found that GBM patients with a low CCL2 expression survived significantly longer than those with a high CCL2 expression. These findings raise the question as to whether reducing monocyte infiltration by targeting the CCL2–CCR2 axis is a viable option for treating murine PDGFB–GBM, considering that 80% of the TAMs in this subtype are of monocyte origin. To address this question, we showed that genetically interrupting the CCL2–CCR2 axis prolonged the survival of GBM-bearing mice, in agreement with previous pharmacological studies (56, 57). However, in contrast to the promising preclinical studies, neutralizing monoclonal antibodies against CCL2 administered to patients with metastatic, solid tumors did not produce favorable outcomes. Although a similar treatment has not been applied to GBM clinically, caution should be exercised if such an approach is to be considered, because different GBM subtypes maintain different compositions of infiltrating TAMs. Tumors with low levels of bone marrow-derived TAMs may not respond to this therapy. This critical point is also reflected by the fact that anti-VEGFA antibody worked only in the PN subtype when combined with RT, but did not show efficacy in the other GBM subtypes (58).

Microglia relies on colony-stimulating factor 1 (CSF-1) for survival, and CSF-1 receptor inhibitors can effectively eliminate microglia in the brains of naïve mice (35). Although pharmacoactive compounds have demonstrated excellent efficacy in preclinical animal studies against a GEMM of PN GBM, they were not successful in eliminating or decreasing TAM numbers in GBM, suggesting that TAMs gain CSF-1 independence (59). However, a CSF-1 receptor inhibitor failed to provide clinical benefit in non-stratified recurrent GBM patients (60). This failure in translation is likely because TAM heterogeneity was not sufficiently addressed and that there is still a lack of knowledge regarding their differential composition and functions as discussed above. It may also suggest a differential role of CSF-1 in human versus mouse. In order to develop effective therapies, it is paramount that we understand the unique functionalities of TAMs in individual GBM subtypes. RNA-seq analyses of purified populations can provide insights into the pathobiological attributes in tumor development as well as subtype-specific differences.

As discussed above, TAMs are highly plastic and maintain the capability to switch between the tumoricidal M1 and tumorigenic M2 phenotypes. Efforts have been made to achieve "re-education" of TAMs to polarize them toward M1. Nanoparticles, for example, can effectively penetrate solid tumors and locally deliver a drug. Nanoparticles carrying IL-12, which is a Th1-polarizing cytokine, can promote the reversal of TAMs from M2 to M1 (61). To move one step further, it was recently shown that intratumoral delivery of oncolytic virus expressing IL-12 along with systemic administration of anti-CTLA-4 and anti-PD-1 antibodies can significantly prolong the survival of GBM-bearing mice. This beneficial effect was primarily attributed to the M1 polarization of TAMs upon therapy. However, it is interesting to note that the depletion of CD4 T cells can eliminate this therapeutic effect, presenting a previously unappreciated link between TAMs and CD4 T helper cells, as well as tumor death (62).

IMMUNE CHECKPOINTS AND THEIR INHIBITORS IN GBM

Immune checkpoints refer to negative regulatory pathways that function to inhibit T-cell activation and proliferation, thereby maintaining self-tolerance and limiting the duration and amplitude of immune responses (63). Cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4), programmed cell death-1 receptor (PD-1), and T-cell inhibitory receptor (TIM-3) are often found on T cells to perform inhibitory functions through interactions with their corresponding ligands (**Figure 3**). Studies using PD-1 knockout mice demonstrated that PD-L1 in T cells, antigen-presenting cells (APCs), and host tissue negatively regulated T-cell response (64). Furthermore, in mice, it has

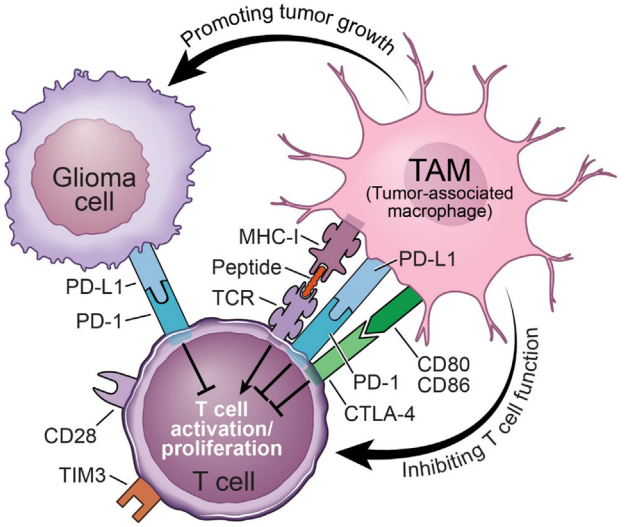

FIGURE 3 | Mechanisms of tumor-associated macrophages (TAMs) inhibiting the functions of tumoricidal T cells in glioblastoma.

been demonstrated that PD-1 is highly expressed by effector T cells during chronic viral infections. By interacting with its ligand PD-L1, which is expressed by stromal cells such as APCs, PD-1 delivers an inhibitory signal to T cells to attenuate their proliferation and effector functions, which can be reversed by using PD-L1-neutralizing antibodies (65). The presence of PD-1 on the surface of these T cells also serves as an indicator of functional exhaustion (65). These and many other elegant discoveries regarding immune checkpoint inhibitors have entered the field of oncology [for a detailed description, see Ref. (66)]. Tumors have evolved to abduct this system for their own benefit by co-opting the cells in the microenvironment, e.g., TAMs, to express high levels of PD-L1. It was recently documented that both the number of PD-1$^+$ tumor-infiltrating lymphocytes and PD-L1 expression are significantly increased in GBM, providing a rationale for the use of immune checkpoint blockade to interrupt the PD-1/PD-L1 axis as a potential therapy for GBM (67–69). Even though the data on the expression of PD-1/PD-L1 in GBM patients are largely correlative based on immunohistochemical antibody staining or TCGA data mining (67, 70, 71), they nevertheless represent the first steps forward in a new area of research in the GBM field, which is to understand the biological function of PD-1/PD-L1, T-cell infiltration and function, and their interaction with TAMs.

Immune checkpoint inhibitors such as anti-CTLA-4 antibody (Ipilimumab) and anti-PD-1 antibodies (Nivolumab or Pembrolizumab) were approved by the FDA for the treatment of non-resectable or metastatic melanoma and have been successful. However, monotherapy with anti-PD-1 or combinational therapy with anti-PD-1 and anti-CTLA-4 antibodies for the treatment of recurrent GBM recently failed in phase III clinical trial (72). This failure in translation implies that the simple blockade of immune checkpoints may not restore the tumoricidal functions of T cells, which may be intrinsically impaired or exhausted. For instance, it has been found that PD-1 expression on CD4 cells identifies a dysfunctional subset refractory to rescue with PD-1 blockade, suggesting that the influence of immune checkpoint inhibitors may involve the recovery of function in the PD-1–CD4$^+$ T-cell compartment (73). It may also imply yet again that GBM should not be viewed as a single entity, but rather a complex tumor of molecular subtypes, which may not respond equally to a given therapy. Indeed, about 8% of all patients in this trial responded well to Nivolumab (72). Although their molecular subtypes have not been characterized, it is possible that these patients maintain a molecular commonality that is sensitive to this therapy. In addition, the functional significance of PD-1/PD-L1 blockade should be evaluated beyond correlative studies.

TIM-3 is enriched in GBM and IDH-wild-type gliomas. TIM-3 is a protein encoded by the *HAVCR2* gene that mediates T-cell-mediated immune functions such as the response to tumor cells and cytotoxicity directed against tumor cell targets. It also mediates similar inflammatory activation functions as PD-L1 in glioma. Interestingly, TIM-3 is a potential marker for the MES molecular subtype. Clinically, the high expression of TIM-3 has been shown to be an independent indicator of poor prognosis. All of these factors make TIM-3 a potential focal point for immunotherapeutic strategies when gliomas gain resistance to antibodies against PD-1/PD-L1 (74).

CONCLUDING REMARKS

Recent advances in cancer immunotherapy have created great enthusiasm and anticipation for an effective treatment for GBM. Most of the current cancer immune therapies, however, focus on the importance of cytotoxic T cells. This may undervalue the significance of innate immune components in the tumor microenvironment, such as TAMs. Tumors are highly adaptive and maintain abundant non-neoplastic cells; therefore, concomitant therapies involving multiple aspects that simultaneously target tumor cells, TAMs, and T cells should be considered. In this regard, it has been shown that blocking TAM-mediated immunosuppression holds great promise for increasing the efficacy of gene therapy-mediated immunotherapies for GBM (75). Further, considering the robust differences in molecular signaling, TAM composition, and T-cell abundance between GBM subtypes, combinatorial therapy with subtype-specific considerations could yield greater success for future GBM immunotherapies.

AUTHOR CONTRIBUTIONS

Both ZC and DH contributed to conceive the article, review the literature, and write the manuscript.

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REVIEW
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Lifelong Impacts of Moderate Prenatal Alcohol Exposure on Neuroimmune Function

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In utero alcohol exposure is emerging as a major risk factor for lifelong aberrant neuroimmune function. Fetal alcohol spectrum disorder encompasses a range of behavioral and physiological sequelae that may occur throughout life and includes cognitive developmental disabilities as well as disease susceptibility related to aberrant immune and neuroimmune actions. Emerging data from clinical studies and findings from animal models support that *very low to moderate levels* of fetal alcohol exposure may reprogram the developing central nervous system leading to altered neuroimmune and neuroglial signaling during adulthood. In this review, we will focus on the consequences of low to moderate prenatal alcohol exposure (PAE) on neuroimmune interactions during early life and at different stages of adulthood. Data discussed here will include recent studies suggesting that while abnormal immune function is generally minimal under basal conditions, following pathogenic stimuli or trauma, significant alterations in the neuroimmune axis occur. Evidence from published reports will be discussed with a focus on observations that PAE may bias later-life peripheral immune responses toward a proinflammatory phenotype. The propensity for proinflammatory responses to challenges in adulthood may ultimately shape neuron–glial-immune processes suspected to underlie various neuropathological outcomes including chronic pain and cognitive impairment.

Keywords: glia, inflammation, cytokines, spinal cord, neuropathy

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INTRODUCTION

Exposure to alcohol during gestation can lead to a constellation of mild to severe disabilities that include cognitive (i.e., intellectual ability, learning, and memory) and behavioral (e.g., mood, attention, and impulse control) sequelae representing a continuum referred to as fetal alcohol spectrum disorder (FASD). Despite nearly 40 years of clinical studies and research in animal models demonstrating *in utero* alcohol exposure acts as a teratogen with very broad and long-term adverse effects, a recent study estimates that globally, about 10% of women in the general population consume alcohol during pregnancy. In discrete regions/countries, the percentage of women who consume alcohol while pregnant is much more (~46%) (1, 2).

While the most profound and most widely known consequences of prenatal alcohol exposure (PAE) encompass clearly identifiable neurobehavioral outcomes, more recent reports are uncovering PAE's far more subtle and insidious lifelong effects on neuroimmune function. Studies examining altered neuroimmune responses as a consequence of PAE are shedding light on potential underlying molecular mechanisms associated with PAE-induced neurological dysfunction (3–6). In studies utilizing animal models of PAE, robust neuroimmune activation such as heightened proinflammatory

cytokine production is observed in the neonatal and adult brain (3, 4, 6–12). Often, these animal models include high and/or chronic prenatal and neonatal alcohol exposure to mimic the effects of binge/heavy drinking during and after (during lactation) pregnancy observed in humans. Additionally, albeit more sparse, clinical and controlled animal studies have been conducted to address whether comparatively less frequent or moderate gestational alcohol exposure exerts similar effects on neuroimmune function. In this review, the focus is predominantly on studies modeling moderate or low PAE and the effects of this exposure on the neuroimmune axis. In this context, discussion points will highlight the possible role PAE may play in shaping the inflammatory response in the central nervous system (CNS).

FASD SPANS A RANGE OF DEFICITS FROM SEVERE TO MILD

Early Clinical Observations of Fetal Alcohol Syndrome (FAS) and the Evolution of FASD

Birth anomalies resulting from PAE were first described as FAS in 1973 by Jones and colleagues (13, 14). Since these seminal reports, the FAS criteria and diagnostic schemas have been refined. FAS is considered the most serious consequence of high levels of PAE and is distinguished from less overt outcomes that are encompassed by FASD. FAS can include significant pre- and postnatal growth delays and a characteristic pattern of craniofacial abnormalities. Additionally, FAS-associated defects have been observed in a variety of organ systems such as the visual, auditory, cardiac, and urogenital systems (15). However, since the first description of FAS, it has become profoundly clear that not all individuals exposed to high levels of prenatal alcohol reveal overt dysmorphia. Other less clearly defined neuropathological conditions inclusive of cognitive and behavioral deficits are now recognized outcomes of FAS. The range and magnitude of cognitive and behavioral deficits vary, which are likely influenced by many factors including alcohol exposure experienced during discrete developmental periods, mother's alcohol consumption pattern, the nutrition status of the mother, as well as genetic factors (16–19). Therefore, the umbrella term FASD has been developed to better capture the complexity (e.g., magnitude and pattern of alcohol consumption) and wide-ranging consequences of PAE that includes the more severe FAS. Notably, FASD encompasses various diagnostic conditions that not only capture FAS, but also partial FAS, alcohol-related neurodevelopmental disorder, and alcohol-related birth defects (20, 21). The neurological sequelae present in individuals across the spectrum of FASD now incorporates cognitive impairments such as deficits in learning and memory, executive and motor function, attention and behavioral problems inclusive of psychiatric and substance abuse disorders, and diminished skills related to social interaction (22). Moreover, a number of studies suggest that the effects of FASD may alter bodily systems such as the immune system that is known to impact neurological function.

Although immune dysfunction is not considered diagnostic of FASD, multiple clinical reports and case studies indicate that children with FASD frequently face secondary medical disabilities related to immune dysregulation (i.e., autoimmune or inflammatory reactivity). For example, FAS children have high rates of upper respiratory infection and recurrent serious otitis media (middle ear inflammation). Additionally, children diagnosed with FAS and neonates prenatally exposed to alcohol experience a high incidence of infection and immune-related pathologies, such as urinary tract infection, meningitis and the chronic autoimmune neuromuscular disease, myasthenia gravis (23). Furthermore, maternal alcohol consumption increases circulating proinflammatory cytokine exposure to the fetus (24). Because alcohol levels persist longer in the blood of the fetus than in the mothers' (25), it is possible that circulating fetal proinflammatory cytokines from alcohol exposure reprogram inflammatory responses long after birth. PAE with consequent increased fetal proinflammatory cytokine levels may underlie some of the cognitive impairments seen with FASD, as discussed further below. However, human studies assessing CNS-specific neuroimmune parameters in FASD individuals are rare due to limited methodologies and access to samples. The majority of human clinical studies of adverse neurological outcomes and aberrant immune competence are from self-reports of heavy drinking mothers or mothers with children diagnosed with FAS.

Human Moderate PAE Studies

A surprisingly limited number of human studies address the causal effect of moderate drinking (i.e., 1 standard drink or 14 g of ethanol per day) during pregnancy and later outcomes of these children (26). Of the few clinical studies aimed to address this important public heath question, a recent prospective cohort study was conducted that included women who consumed light to moderate alcohol levels that did not report binge drinking. The behavioral trajectories of the children were followed. This study reported that the children of mothers' who reported moderate drinking during pregnancy experienced an increased risk for early childhood behavioral problems (e.g., getting along with other children in a group) (27). A comprehensive systemic review by Mamluk et al. concluded that due to the paucity of well-designed human studies, it remains unclear whether a safe limit of alcohol consumption during pregnancy exists (28). More recently, Muggli et al. suggested that even low levels of PAE can influence craniofacial development, thus supporting that abstinence from alcohol while pregnant is the safest option (29). In an attempt to address the gap in knowledge of the clinical CNS developmental consequences from low/moderate PAE, a number of well-controlled studies applying animal models have been reported. Indeed, the application of low to moderate PAE in animal experiments has significantly advanced our understanding of the potential direct and indirect effects of PAE on the CNS inflammatory response.

ANIMAL MODELS OF PAE REVEAL BASAL NEUROIMMUNE CHANGES

A number of published reports have modeled the impact of moderate PAE on the developing and adult CNS in animals (12, 26, 30–32). Studies in rodent models demonstrate long-lasting

neurobehavioral deficits caused by moderate levels of alcohol exposure during prenatal (first and second trimester pregnancy) and neonatal (equivalent to the third trimester of human pregnancy) development. Abnormalities in learning, memory, motor coordination, social behavior, and stress responses were observed. Notably, these behavioral alterations are associated with impairments in neurotransmitter systems, neuromodulators, and synaptic plasticity in several brain regions (26). In studies aiming to generate moderate PAE, pregnant dams achieve blood alcohol levels within the range of 0.08–0.17 g/dl (80–170 mg/dl). For reference, 0.08 g/dl is considered the US legal intoxication limit. One aspect of these earlier studies that came to light is the effect of moderate PAE on CNS-immune interactions.

While collective evidence from many animal studies suggest that moderate or low-alcohol exposure can persistently alter multiple neurotransmitter and neuromodulatory systems (26), few studies have demonstrated that moderate PAE affects neuroimmune function. Data have been ambiguous regarding the CNS outcomes from developmental low-dose alcohol exposure with several previous studies suggesting moderate PAE might be less disruptive to neurodevelopment and the neuroimmune system than binge-like exposures (33–35). However, a lack of comparative studies exist that investigate neuroimmune parameters and neurodevelopment following discrete trimester and pattern of alcohol exposure (binge versus moderate). Despite the brevity of needed basic science studies, the following section will discuss evidence to date that point to possible mechanisms underlying the impact that PAE exerts on neuroimmune function during CNS development, the early postnatal period and in adulthood.

Immune reactions within the CNS are initially and predominantly mediated by glial cells, often with engagement of infiltrating innate/adaptive immune cells from the periphery (36). While glial cells (microglia and astrocytes) play key roles in neuronal homeostasis in the CNS, they are also primary immune responders in the CNS (37, 38). Upon immune activation, glial cells in the adult brain secret proinflammatory cytokines such as interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF), cyclooxygenase 2, and nitric oxide as well as anti-inflammatory factors such as IL-10 (39–41). Neuroinflammatory agents from glial cells stimulate neurons and infiltrating immune cells that in turn secrete chemokines including monocyte chemotactic protein-1 [MCP-1, also known as C–C motif chemokine ligand-2 (CCL2)], thereby furthering neuroinflammatory processes that contribute to neuropathologies associated with many degenerative and neuroinflammatory disorders (42–44).

While the animal model for moderate PAE is the primary focus of this review, most of our knowledge of neuroimmune responses following alcohol exposure has come from experiments that model binge-drinking where sporadic and high levels of developmental alcohol exposure occurs. In consideration of these animal models, the data reveal that alcohol exposure during early postnatal development in rodents (a third trimester equivalent in humans) results in microglial and neuronal loss, microglial, and astrocyte activation (7, 45) and increased proinflammatory cytokine and chemokine expression in diverse brain regions such as the hippocampus, cerebellum, and cerebral cortex (4). Moreover, neuroinflammation in the fetus has been verified (46, 47). These results suggest a link exists between glial function and ethanol-induced neuropathology. Indeed, other studies reveal that the adverse effects of alcohol on CNS glial activation are mitigated by an anti-inflammatory agent, a peroxisome proliferator-activated receptor gamma agonist (4, 7) that blocks microglial activation.

A recent report by Pascual et al. examined the neuroimmune effects of *moderate* prenatal and postnatal alcohol exposure. In this model, pre-pregnant female breeder mice received 10% ethanol (v/v) in their drinking water for 2 months before mating resulting in an average peak blood alcohol concentration (BAC) of 125 ± 20 mg/dl. After 2 months, these pre-pregnant females were placed with male breeders, and following pregnancy, dams continued to receive 10% ethanol solution throughout gestation and lactation, with weaning occurring at postnatal day (PND) 25 (48). Tissues were collected from discrete subgroups representing specific timepoints. Cerebral cortices were collected at embryonic day 15 (E15), as well as on PND 0, 20, and 66 followed by an examination of key proinflammatory cytokines and chemokines. This study demonstrated increased IL-1β, IL-17, macrophage inflammatory protein (MIP)-1α, and fractalkine protein levels in fetal cerebral cortex at E15 (48). In this study, sex differences were not determined at this timepoint. These cytokines remained elevated at birth (PND 0), along with increased CCL2 (MCP-1), but returned to normal levels by PND 20 in females; however, the pattern observed in males is distinctly different, as discussed below. Additionally, IL-1β protein levels remained consistently and significantly elevated through PND 66, as cortex was examined at E15, PND 0, 20, and 66. Increased protein levels of CD11b (integrin alpha M chain) and major histocompatibility complex 2 (MHC2) (at PND 0 and 20) were also detected in the brain (48), suggesting an increase in myeloid cell activation/activity.

However, as alluded to above, interesting sex differences were observed in these neuroinflammatory effects, with increased levels of MCP-1 and MIP-1α only detected at PND 20 in male pups without an increase at PND 0, suggesting the basal postnatal expression of these chemokines is influenced by as yet unrecognized postnatal gender-specific factors, with cytokine expression patterns programmed during prenatal development. In support of this possibility, increases in female-derived CCL2 and MIP-1α protein levels were observed significantly sooner (PND 0) but, plummeted by the third week of postnatal development (PND 20) compared with levels observed in males. Additional sex differences in cytokine levels, as no changes in IL-1β levels were measured in male pups with pre- and postnatal alcohol exposure. Overall, these sex differences were associated with increased neuroinflammation in female pups in this particular model of pre- and postnatal alcohol exposure (48).

In a third model of PAE, pregnant dams were administered alcohol (using a flexible gavage catheter, 2 g/kg of ethanol, twice daily) only during gestational days 10–16, with dam's peak BACs reaching 70 mg/dl (12). This study showed robust increases in cytokines and chemokine/chemokine receptors such as CCL2, 3, and 6, C–C motif chemokine receptor 2 (CCR2) (the receptor for CCL2), CCR6, IL-21, IL-10, and TNF mRNA in the fetal hippocampus and cortex at E17. An additional important finding

from this report demonstrated that the alcohol-induced effects on the maternal immune system appear to be minimal, while the placenta and the developing fetal brain mount rapid (~24 h) and robust immune responses as a consequence of moderate PAE (12). These results from PAE models are further supported by other reports where animal studies of perinatal cytokine and chemokine increases within the fetal brain co-occur with impaired neonatal brain white matter microstructural integrity as well as motor dysfunction in offspring (49, 50). Combined, these reports suggest that elevated circulating proinflammatory factors (elicited either by immune stimulation or by PAE) during pre- and perinatal periods may significantly contribute to brain neuropathology in young offspring.

Intriguingly, these seminal reports on the impact of PAE on neuroimmune function revealed distinctly different observations of neuroimmune responses between males and females. Specifically, Terasaki and Schwarz (12) reported that acute (~24 h) neuroinflammatory gene activation occurring in response to low levels of PAE during early fetal brain development are sex specific. For a number of immune molecules such as CCL2 and IL-5, the effects of PAE were dependent on the sex, because their expression levels were generally decreased in males but increased in females with PAE. Overall, female pups (E17) with PAE revealed higher levels of inflammatory gene expression in the brain compared with their male counterparts (12).

In addition to the PAE sex-specific differences in chemokines/cytokines, the same report demonstrated increased levels of CD11b and brain-derived neurotropic factor in adult brain examined on PND 90 (51). These data indicate that long-term immune activation in different brain regions does not require a parallel induction of cytokines. In a separate study utilizing moderate PAE, increases in adolescent hypothalamic CCR2 receptor was reported (10). Observations from our laboratory demonstrate that low-dose alcohol exposure throughout gestation in rats (peak dam BAC ~60 mg/dl) resulted in changes in immune cell markers in adult offspring. For example, increased expression of β-integrin adhesion molecules (e.g., CD11a and CD29) on $CD11b^+$ cells collected from spinal cord was measured. Increased expression of these adhesion molecules may reflect altered/impaired regulation and/or hypersensitivity to immune stimuli by immune cells and/or microglia (52).

As noted above, greater levels of alcohol exposure during early postnatal development in rodents (a third trimester equivalent in humans) results in microglial and astrocyte activation and microglial loss (7, 45). However, microglial *loss* has not been reported following *moderate* PAE. Rather than microglial loss, published data show moderate PAE induces early neuroinflammatory responses in the fetal brain, and while speculative, early developmental CNS inflammation may affect the pre- and postnatal roles of microglia on neuronal development. Moreover, PAE-induced activation of microglia may cause persistent changes in their activation status and function resulting in neuroimmune and neurobehavioral consequences following a subsequent challenge (immune or tissue damage) in adulthood. In support of this possibility, a growing body of evidence, as discussed in the following sections highlights the potential long-term impact of moderate PAE in shaping CNS responses to subsequent challenges.

MODERATE PAE SHAPES NEUROIMMUNE RESPONSES TO SUBSEQUENT INFLAMMATORY CHALLENGE LATER IN LIFE

CNS Immune Responses Upon Brain Injury

While speculative, glial cells may remain primed following an initial stimulation during gestational development (i.e., PAE), with classic activation markers returning to basal levels. However, these previously stimulated glia may develop an increased propensity for enhanced responses following subsequent immune stimulation (52, 53). That is, it is possible under some circumstances that classic glial activation markers are uncoupled from their primed state such that basal levels of these markers are observed, yet these same glia over-respond to normal stimuli. For example, PAE primes the CNS glial-immune response as observed by enhanced inflammatory cytokine production following subsequent immune challenge during adulthood. The observation that fetal exposure to alcohol alters responses of glial and immune cell factors to CNS injury in adults was first described by DeVito and Stone (54). In this study, animals were exposed to moderate levels of ethanol (46–101 mg/dl) *in utero*. The PAE offspring were maintained until adulthood and underwent discrete cortical damage *via* a stab injury. Data show increased vascular cell adhesion molecule-1 (VCAM-1) and cluster of differentiation 68 (CD68) on both microglia and macrophages in PAE animals, indicating brain endothelial cell activation in addition to microglia and/or macrophage activation. Interestingly, glial fibrillary acidic protein (GFAP), a marker of astrocyte activation, and proinflammatory TNF production were diminished in PAE rats compared with the experimental controls in this brain region 4 days following injury. However, re-exposure to alcohol several days before and after the stab wound surgery further augmented CD68 and VCAM-1 expression in adult brains of PAE animals (54). These data suggest moderate PAE has the potential to alter and augment key components of neuroimmune responses to subsequent CNS injury in adulthood.

Neuroimmune and Cognitive Outcomes in PAE Adults Following Challenge With LPS

The hippocampus and cortex are critical brain regions for learning and recognition memory. These brain regions are also vulnerable to glial and immune activation (55). In the study by Terasaki et al., moderate PAE rats were given a systemic challenge on PND 90 with a low dose of lipopolysaccharide (LPS; 25 μg/kg, intraperitoneal injection) to induce an acute immune response. Notably, LPS expresses pathogen-associated molecular patterns signaling to immune cells that pathogen invasion has occurred. Within 4 h of LPS injection, exaggerated IL-1β and IL-6 protein levels were measured in the hippocampus of PAE male rats. In the medial prefrontal cortex (mPFC), PAE and LPS immune challenge generated additive IL-1β increases in both males and females. Greater levels of CD11b protein were also detected in adult mPFC of PAE males and females, while no changes were detected in the

hippocampus (12). These proinflammatory cytokines are thought to play significant roles in cognitive and psychiatric disorders (56–58). Thus, cognitive function in adult PAE rats with adult-onset immune activation (with LPS) was investigated. Several important outcomes were observed from this report. First, data revealed that basal PAE (without LPS challenge) reduces performance on recognition memory tasks [e.g., novel object recognition (NOR)] in adult males and females. Second, upon mild immune activation in adulthood, profound recognition memory deficits were observed in PAE offspring (both males and females) (12).

In a more recent study, PAE rats were further exposed to an acute binge-like dose of alcohol in adulthood (51). This study revealed that PAE exaggerated IL-6 production in the prefrontal cortex following alcohol exposure in adulthood, suggesting that adult alcohol exposure may act as a neuroinflammatory agent. Interestingly, IL-1β and CD11b levels in the cortex were decreased in response to acute adult binge-like alcohol exposure regardless of PAE. In this same study, adult cognitive function was assessed by performance on hippocampal-dependent (novel object location/NOL) and non-hippocampal-dependent (NOR) behavioral tasks. Adult cognitive function was disrupted in PAE offspring with or without adult alcohol exposure. Minor sex differences were additionally observed. Females (with PAE alone) and males with PAE in combination with an acute binge-dose of alcohol were deficient in learning the NOL task. In addition, male PAE animals and PAE plus an acute binge-like dose of alcohol in adulthood (in both males and females) negatively impacted performance the NOR task (51). Together, these data highlight that low/moderate levels of PAE can negatively impact cognitive function in later life, and these long-term consequences can be exacerbated by subsequent challenges (e.g., binge-like doses of alcohol exposure or immune activation).

SPINAL GLIAL-IMMUNE RESPONSES THAT ARE ALTERED BY MODERATE PAE: INCREASED VULNERABILITY TO CHRONIC NEUROPATHIC PAIN

Multiple studies now suggest that even low levels of PAE may pathologically prime CNS glial cells and disrupt their supportive role in neuronal function, not only during development, but also throughout adulthood. PAE may generate CNS susceptibility to injury through the actions of aberrant immune responses that ultimately act to exacerbate challenges in the CNS rather than sequester damage and enhance healing. Therefore, we hypothesize that the risk and severity of chronic neurological disorders is enhanced as a consequence of PAE. In recent years, our laboratory has examined the development of adult-onset chronic neuropathic pain in an animal model of moderate PAE to better elucidate the spinal cellular and molecular neuroimmune adaptations PAE induces.

PAE Potentiated Spinal Immune Responses and Chronic Pain

An intriguing behavioral manifestation in children diagnosed with developmental disabilities including FASD is abnormal sensory function such as tactile hypersensitivity (59–63). In support of clinical observations, a longitudinal study in rhesus monkeys revealed that heightened sensitivity to light touch was significantly greater in adult PAE monkeys compared with control-treated monkeys (30). Curiously, tactile hypersensitivity is also frequently observed in people with peripheral nerve damage and is referred to as allodynia. From the perspective of pain transmission, allodynia results from aberrant synaptic communication in the spinal cord where incoming sensory transmission is relayed to higher brain areas. Thus, allodynia following peripheral nerve damage is a CNS disorder. Therefore, one hypothesis that quickly developed was whether the underlying cause for tactile hypersensitivity (allodynia) observed in individuals with FASD could be due to neurological dysregulation of spinal pain relays. Furthermore, many reports utilizing rodent models of peripheral nerve damage demonstrate that allodynia occurs as a consequence of over-active spinal astrocyte and microglial responses (39).

As noted, a large body of evidence demonstrates that the neuro-glial-immune interface may underlie aberrant adult CNS function as a consequence of PAE. This background pointed to the hypothesis that PAE may exacerbate allodynia through elevated spinal glial actions. Therefore, a well-characterized rat model of low/moderate PAE (64) was utilized to determine whether enhanced allodynia and immune and glial responses occurred following peripheral nerve challenge in adult PAE offspring (52). The data indeed suggest several overlapping neuroimmune interactions are present between chronic pain and PAE. **Figure 1** summarizes the proposed spinal mechanisms by which PAE potentiates chronic neuropathic pain based on current published reports.

A brief overview of chronic pathological pain will provide the appropriate context supporting the rationale for studies that explored spinal glial mechanisms underlying enhanced pain in PAE offspring. Chronic pathological pain often results from peripheral nerve damage, infection, or a combination of both trauma and inflammation. While chronic pathological pain involves hyperexcitability (sensitization) of pain projection neurons in the spinal cord or brainstem, critical roles of glial cells in the spinal cord, and satellite glial cells of the dorsal root ganglia (DRG) have been established (65, 66). Following injury of peripheral axons (e.g., sciatic nerve), excitatory pain transmitters are released by the nerve terminals projecting to the spinal cord that, in turn, synapse onto pain projection neurons. As noted above, the surrounding glial cells (**Figure 1A**) respond to these classic pain transmitters. The glial response includes release of IL-1β and TNF, and the chemotactic cytokine, CCL2/MCP-1 among a number of proinflammatory factors. Increased CCL2–CCR2 actions upon neuronal and glial activation in the spinal cord and DRG following peripheral nerve damage can result in leukocyte accumulation in discrete anatomical regions along the pain pathway (e.g., peripheral nerve axons, DRG, corresponding spinal cord regions, **Figure 1B**) (67–71). Over time, due to the feed forward actions of IL-1β and TNF and other cytokines released from activated glia and infiltrating immune cells, pathological sensitivity to non-painful stimuli develops (e.g., mechanical allodynia) resulting in chronic neuropathic pain (67, 72).

The crucial contributions of both protective and detrimental roles of active glial cells during conditions that lead to chronic painful neuropathy (39) necessitated further exploration of PAE-

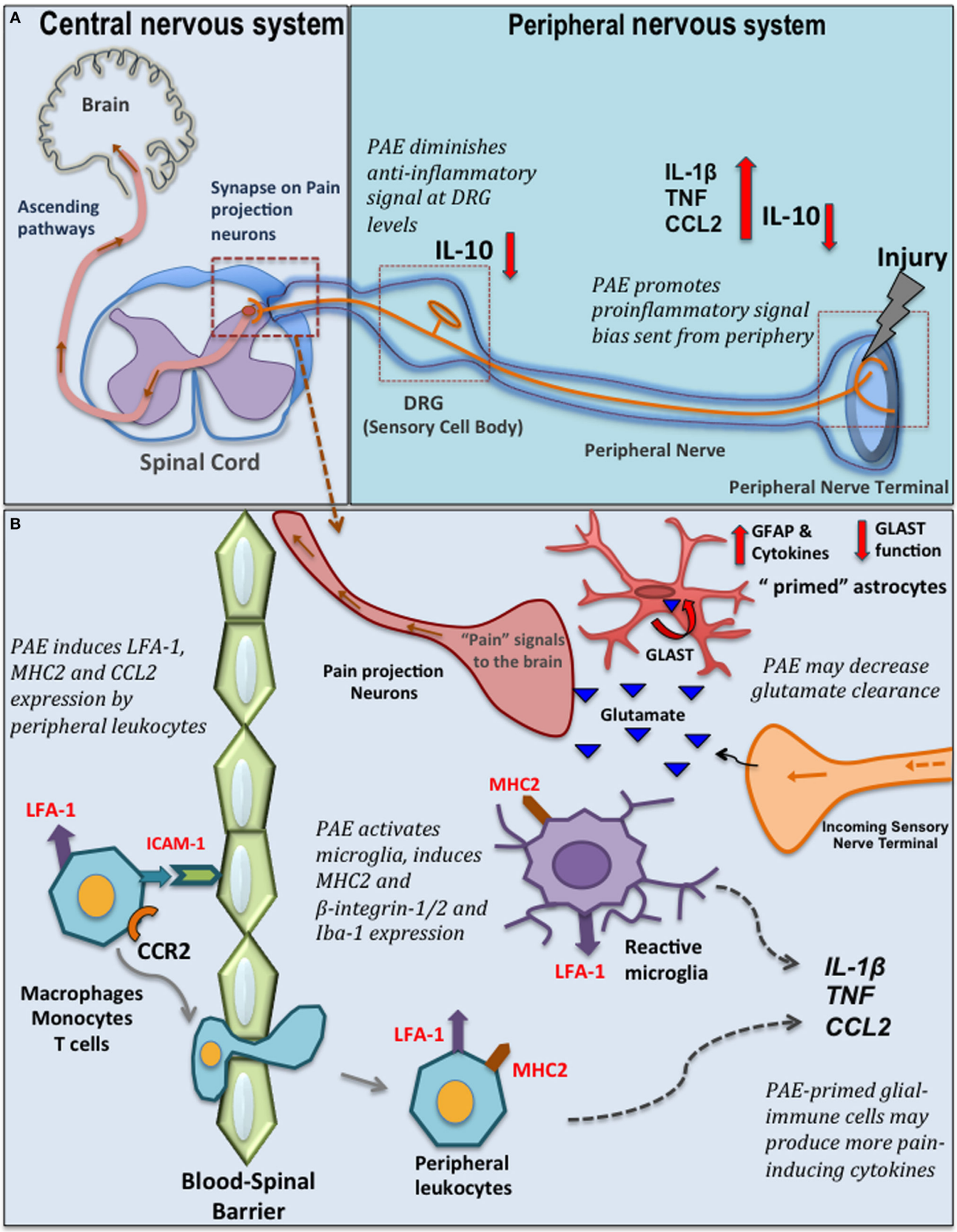

FIGURE 1 | Continued

FIGURE 1 | Prenatal alcohol exposure (PAE)-induced changes in spinal glial-immune interactions during chronic pain. **(A)** Peripheral nerve injury activates resident macrophages and Schwann cells that send out the danger signals, such as nitric oxide and chemokines to recruit peripheral immune cells (e.g., macrophages, mast cells, neutrophils, and T cells) that invade the lesioned site. These immune cells produce further proinflammatory cytokines/chemokines such as CCL2, tumor necrosis factor (TNF), interleukin (IL)-1β. Eventually, a compensatory increase of IL-10 is observed that dampens the peripheral inflammatory reactions at the lesion site. In addition, significant pain modulation and spinal glial-immune activity occurs at the level of the dorsal root ganglia (DRG) (where sensory neuron cell bodies are located) and spinal cord. Following nerve injury, satellite glial cells and infiltrating peripheral immune cells in the DRGs produce cytokines and further modulate sensory neuron (nociceptors relay painful noxious stimuli) activity and gene expression. Simultaneous significant microglia and astrocyte activation is observed in the dorsal horn where the primary afferent fiber terminals relay pain information to secondary pain projection neurons within the dorsal horn of the spinal cord. Neurotropic factors (brain-derived neurotropic factor) and cytokines produced by glia (satellite glia, microglia, and astrocytes) and peripheral immune cells at these discrete regions significantly modulate pain processing. PAE alters the inflammatory milieu at the peripheral lesion site and at the DRG promoting a bias toward proinflammatory signaling, with a simultaneous significant deficiency in IL-10 production that may contribute to the exaggeration of danger signals relayed from periphery to the central nervous system-immune system. **(B)** PAE augments production of CCL2 by peripheral leukocytes. CCL2–CCR2 mediated interactions may further activates LFA-1 to promote LFA-1-ICAM-1 mediated trans-endothelial leukocyte migration across the blood–spinal barrier. In the spinal cord, these peripheral leukocytes also produce various cytokines and interact with neurons and glial cells to further activate glia. Moreover, PAE is thought to prime glial cells, as evidenced by increased adhesion molecules and major histocompatibility complex 2 (MHC2) expression (52). Therefore, with nerve injury, PAE potentiates microglial and astrocytic activity, which may in turn lead to increased proinflammatory cytokine production in the spinal cord dorsal horn. Also, PAE may alter homeostasis of neurotransmitters (such as glutamate) by decreasing glutamate transporter expression, therefore activating pain projection nerve terminal *via* excessive glutamate. GLAST, glutamate aspartate transporter; CCR2, C–C motif chemokine receptor 2; CCL2, C–C motif chemokine 2; ICAM-1, intracellular adhesion molecule-1; LFA-1, leukocyte function-associated antigen-1; Iba-1, ionized calcium binding adaptor molecule; GFAP, glial fibrillary acidic protein.

induced altered glial responses to peripheral nerve injury. Our recent work elucidates the impact of moderate PAE on spinal neuron–glia-immune interactions using an adult-onset peripheral nerve damage model of neuropathic pain (52). Chronic constriction injury (CCI) of the sciatic nerve is a widely used rodent model of sciatic trauma leading to neuropathy in which 4 snugly tied, but not pinching, chromic gut sutures are applied (73). In this study, CCI was applied to 4–5-month-old PAE male rats (equivalent to young adulthood). The data reveal that PAE potentiates allodynia. Moreover, this work demonstrates that even with moderate PAE, spinal microglia are primed, as indicated by increased CD11b expression. It was also observed that PAE, regardless of peripheral nerve damage, enriched the β2 integrin$^+$ (leukocyte function-associated antigen) and β1-intergin$^+$ and MHC2$^+$ population of microglia and leukocytes in the lumbar spinal cord (52). Expression of these β-integrin adhesion molecules and MHC2 suggests involvement of microglial *and* immune cell activation, migration, antigen presentation, and primed functional responses (74, 75). While these data are indicative of PAE-induced priming of spinal glial and immune cells, further studies are needed to determine whether PAE-induced priming leads to increased activation of β-integrin on peripheral leukocytes (76) that is driven by increased CCL2-mediated interactions in PAE spinal cord.

Additional evidence exists supporting the possibility that PAE augments astrocyte and microglial activation in the dorsal horn of the spinal cord in neuropathic adult male rats. That is augmented expression of Iba-1, a well-characterized microglial activation marker, and GFAP (**Figure 1B**) were observed in PAE rats with CCI (52, 53). In line with these observations, analysis of cytokine levels in sham-treated PAE rats revealed that DRG IL-10 protein levels are remarkably suppressed, with IL-10 suppression greatest in PAE neuropathic rats compared with control-treated rats (52). It is important to note that spinal IL-10 is established to suppress allodynia in rodent models of peripheral neuropathy by blocking the spinal actions of a number of proinflammatory factors (77–80). In addition, prior reports demonstrate that DRG IL-10 protein levels are significantly reduced under neuropathic conditions, with satellite glia being a cellular source of DRG IL-10 (81, 82). Thus, in light of prior evidence, the current data suggest PAE further blunts the protective actions of basal DRG glial-derived IL-10 against proinflammatory actions (52). This work additionally suggests moderate PAE primes spinal microglia and astrocytes such that the response of these cells to subsequent damaged-self signals occurring during Wallerian degeneration from peripheral nerve damage is greatly enhanced. While pain relevant cytokine levels and their specific cellular sources (astrocytes, microglia, different subsets of T cells, and/or macrophages) in the spinal cord from neuropathic PAE rats is still under investigation, these data provide evidence that PAE generates stable, potentially lifelong spinal and peripheral nervous system glial, and immune cell hyper-reactivity following a second insult (e.g., localized sciatic nerve trauma) that is initiated in adulthood.

Susceptibility to Developing Chronic Pain Following Minor Nerve Injury

One of the most compelling aspects of the data from offspring with enhanced neuropathy following moderate PAE is that their baseline sensory (light touch) responses are identical to non-PAE controls (52). This striking observation indicates that the insidious effects of PAE on glial–neuronal and immune responses are masked, and only following a second challenge (e.g., nerve injury or increased cytokine exposure), the adverse effects of PAE on neuroimmune responses are revealed. This observation suggests that neuropathological consequences of low/moderate PAE are not overt, but rather, PAE-related glial priming becomes evident only following a subsequent nerve injury or challenge (12, 52). That is, PAE may a be risk factor for developing chronic pathological CNS conditions in response to minor insults that typically go unnoticed in non-PAE individuals because they are resolved by a healthy neuroimmune response.

In support of the possibility that PAE is a risk factor for adult neuropathy, a recent investigation revealed that PAE renders one susceptible to developing pathological pain induced by a mild peripheral nerve injury, possibly through exaggerated immune and spinal astrocyte responses. PAE rats display potentiated allodynia following CCI (with four sutures) that was initiated in late adulthood (1-year-old rats). However, when CCI is reduced to a minor injury with only a *single* suture, robust allodynia was observed in PAE adults while touch sensitivity in control animals

remained entirely unaltered (53). Surprisingly, neuropathic PAE animals from minor injury did not reveal significant spinal cord *microglial* activation by day 10 after CCI, as revealed by immunoreactivity for Iba-1 and transmembrane protein 119, both markers of microglial proliferation and/or activation, in the dorsal horn of the lumbar spinal cord (53, 83). Conversely, significant increases in *astrocyte* activation, as examined by GFAP immunoreactivity were observed in the spinal cord compared with non-neuropathic control rats. While, it is possible that increased microglial activation occurs earlier or later than the time points examined, these data suggest that spinal astrocytes and not microglia are playing a greater role in mediating the PAE-associated risks for developing allodynia following minor injury. Thus, PAE-induced pathological neuroimmune responses underlying abnormal CNS sensory processing can be unmasked by minor injury (53).

POSSIBLE MECHANISMS UNDERLYING THE IMPACT OF PAE ON THE NEUROIMMUNE AXIS IN ADULTS

While exact mechanisms underlying the long-term impacts of moderate PAE on the CNS and the neuroimmune system are currently under investigation, a summary of several putative mechanisms based on available supporting evidence is discussed below. Additionally, **Figure 2** depicts a proposed working model of the possible long-term alterations of different components of the neuroimmune system due to PAE.

Epigenetics-Steady State Alterations

The term "epigenetic" refers to stable, but potentially reversible alterations of genetic information that result in changes in gene expression, but do not involve changes in the DNA sequence itself (e.g., a lack of genetic mutation). This includes DNA modifications and its regulatory factors such as chromatin structure and actions of non-coding RNAs (ncRNAs) (84). Notably, cell fate specificity and differentiation are often related to epigenetic modification. An epigenetic pattern is closely associated with and responsive to environmental cues. With these properties under consideration, epigenetic modification(s) may meditate negative outcomes as a consequence of adverse *in utero* environmental signals such as alcohol exposure. Epigenetic modification can potentially create long-term reprogramming of gene expression where the initial insult to the fetus is long gone, as observed with FASD.

Emerging clinical evidence of epigenetic modification due to PAE provides a possible mechanism for the enduring effects of PAE (85). For example, an important layer of epigenetic regulation is through ncRNAs such as microRNA (miRNA) and long non-coding RNA (lncRNA). miRNA and lncRNA do not undergo translation, but instead, are involved in various aspects of post-transcriptional modification such as inhibiting mRNA translation to protein, mediating alternative splicing events and promoting post-transcriptional modification of different RNA species. Thus, miRNAs have become an active area of PAE research, specifically in the search for a reliable biomarker for PAE (86). For example, a characteristic miRNA signature was recently identified in plasma collected during pregnancy from moms who gave birth to infants affected by PAE that displayed signs of FASD relative to infants not affected and non-alcohol exposed infants. This study identified increases in eleven miRNAs (e.g., hsa-miR-222 5p, hsa-miR-187-5p, and others) in maternal plasma as potential predictors of worsened outcomes in children with heavy PAE (87).

Few studies exist that link long-lasting alterations in miRNA expression in the brain due to moderate PAE (88, 89). However, while evidence of PAE-related epigenetic modification of immune-related gene expression is sparse, Valles et al., reported that exposure to alcohol (105 ± 45 mg/dl) induced hypermethylation of GFAP (astrocyte structural protein) DNA in fetal brain with a concomitant reduction in GFAP mRNA stability and expression (90). Distinct DNA methylation patterns have also been observed in adolescents and children with FASD (91, 92). Based on the existing and emerging evidence, aberrant DNA methylation, regulation of ncRNA, covalent modification to mRNA, and different ncRNA species may likely play a vital role in the etiology of neuroimmune alterations observed later in life due to PAE.

Toll-Like Receptor 4 (TLR4)—CNS Actions

Like other peripheral immune cells, innate immune cells in the CNS express receptors that recognize pathogen-associated molecular patterns such as TLRs and NOD-like receptors (inflammasome NLRs). Data from *in vitro* and *in vivo* studies support that alcohol-mediated neuroinflammation involves downstream signaling following TLR and NLR activation (93, 94). Chronic ethanol treatment (87–140 mg/dl) in adult female mice can activate TLR4 signaling on glial cells, thereby inducing the production of proinflammatory molecules and the upregulation of both CD11b and GFAP (93). In addition, microglia from adult alcohol-treated mice affect neuronal apoptosis (e.g., increases) *via* TLR4-dependent pathways (95). Furthermore, TLR-deficient mice given chronic alcohol exposure as adults, exhibit fewer alcohol-mediated cognitive and anxiety-associated behavioral impairments than wild-type mice expressing the TLR4 receptor (96). Moreover, relevant to post-transcriptional regulation, chronic ethanol treatment in adult mice leads to epigenetic modifications in different brain regions in a TLR4-dependent manner (96). Therefore, TLR4-dependent signaling has been linked to alcohol-induced neuroinflammation and associated behavioral and cognitive deficits in an adult-drinking animal model (96). Similarly, in a model of moderate PAE, it has been shown that alcohol-induced microglial activation and neurodevelopmental alterations are mediated by TLR4 signaling (48). To date, a gap in knowledge exists addressing whether *in utero* exposure of *moderate* alcohol alters TLR and NLR expression and/or function specifically on glial/immune cells. While speculative, it is possible that moderate *in utero* alcohol exposure alters TLR functional responses and their co-receptors (e.g., CD14-co-receptor of TLR4), rendering glia and peripheral immune cells highly responsive to further immune activation with consequent augmentation of CNS proinflammatory responses.

Altered Blood-Brain Barrier (BBB)/ Spinal-Barrier Permeability

Another possible mechanism by which moderate PAE may enhance neuroinflammation is via modification of BBB function, resulting in increased permeability. A fundamental

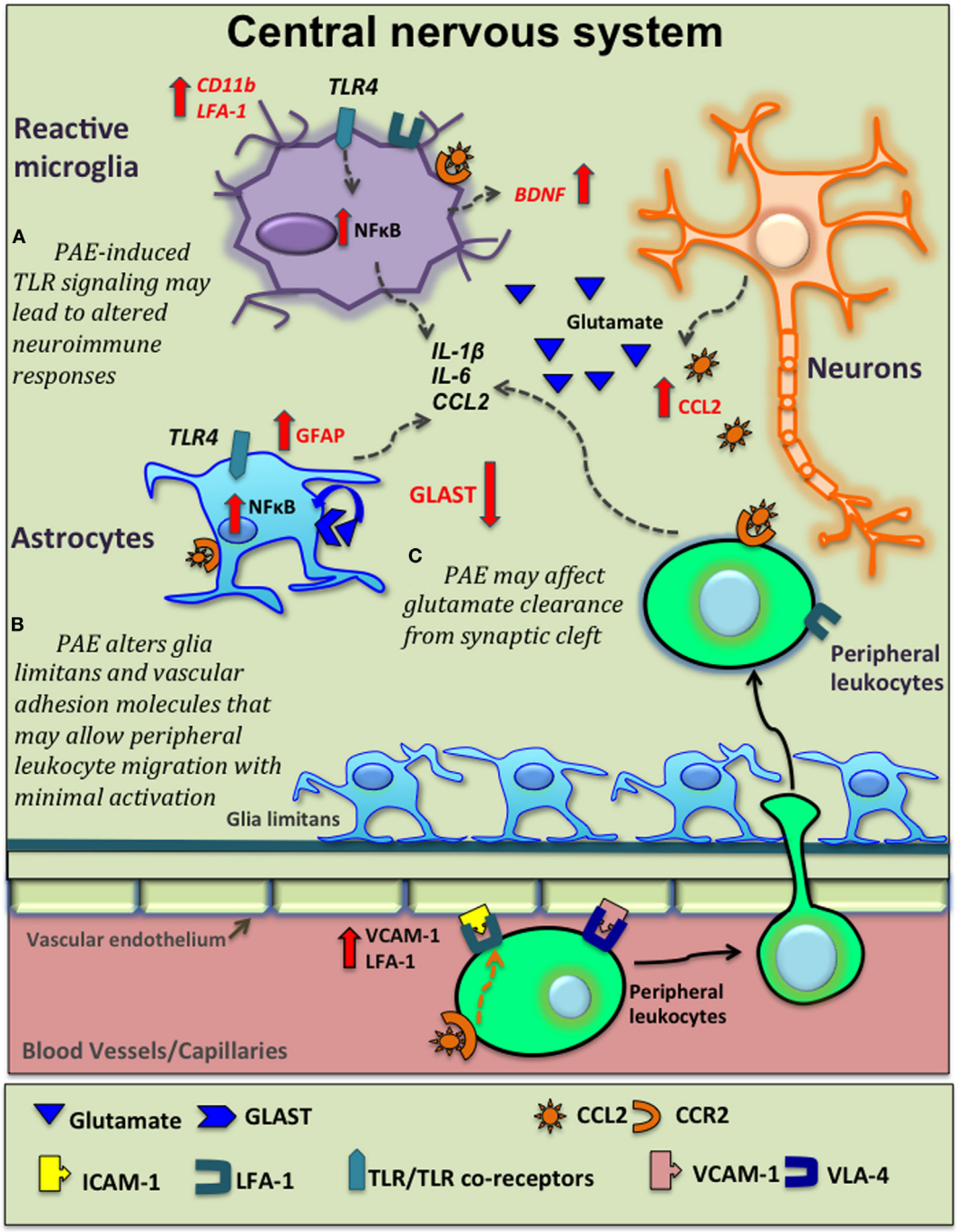

FIGURE 2 | Potential venues of prenatal alcohol exposure (PAE) altering neuroinflammation. Moderate PAE leads to epigenetic alterations of neural gene expression that may extend to glial-immune cells altering their basal activation status and function (not shown here). Based on current supporting evidence, this schematic diagram shows several other potential mechanisms underlying PAE's contribution to central nervous system (CNS) inflammation. **(A)** PAE-induced toll-like receptor 4 (TLR4)-mediated signaling activates the NFκB pathway in microglia and astrocytes leading to production of various inflammatory cytokine, as explored in high-alcohol exposure models. PAE-induced expression and function of TLRs may lead to altered neuroimmune responses followed by subsequent immune activation. **(B)** PAE increases C–C motif chemokine ligand-2 (CCL2) production in the CNS that may be released damaged neurons in the CNS and also *via* blood-brain-barrier (BBB). CCL2 acts on peripheral leukocytes to recruit them to the CNS. Glial cells also express C–C motif chemokine receptor 2 (CCR2) and produce inflammatory cytokines in response to CCL2. Similarly, PAE-induced increases in adhesions molecules such as vascular cell adhesion molecule-1 [VCAM-1, binds with very late antigen-4 (VLA-4) on leukocytes] and leukocyte function-associated antigen-1 (LFA-1), in conjunction with altered morphology of glia limitans, may facilitate and increase the magnitude of peripheral leukocyte migration across BBB following subsequent immune activation. **(C)** PAE decreases glutamate aspartate transporter (GLAST) expression and/or function, decreasing normal glutamate removal by astrocytes, with consequent increased neuronal activation, augmenting pathological neuroimmune interactions.

component of the BBB is the neurovascular unit, which is made up of neurovascular endothelium, basal lamina, pericytes, and astrocytic end-feet. During inflammation, activated glial cells release factors that further activate each cellular component of the neurovascular unit. The BBB is also responsive to circulating peripheral cytokines and oxidative stress, which alert glial cells residing in the CNS of either pathogen invasion or tissue damage (97). Therefore, integrity of the BBB and the neurovascular unit can shape the course of CNS inflammation (98). As of yet, a lack of clear evidence exists confirming whether moderate PAE alone (i.e., without a later-life insult or challenge) underlies peripheral leukocyte infiltration of the adult CNS. However, one report demonstrated that PAE rat offspring whose mothers' achieved average serum ethanol concentrations of 140 mg/dl displayed altered morphological development of the glia limitans, a structure consisting of astrocyte end-feet in contact with the pia mater and capillary endothelial cells (99). This study concluded that PAE might induce defects in the glial limitans resulting in leptomeningeal heterotopia. In a separate study, PAE was found to upregulate the vascular cellular adhesion molecule, VCAM-1 in the brain (54). We propose the tentative argument that PAE may cause subtle leakiness at the neurovascular unit leading to chronic low-level glial reactivity. In turn, low-level gliopathy may allow the BBB to reach a threshold of permeability more readily (even with mild immune activation) thereby facilitating greater CNS leukocyte trafficking following subsequent immune challenges (**Figure 2**). Indeed, the notion of increased leukocyte trafficking was observed following peripheral nerve damage in PAE adults (52). However, it is important to consider that pericytes and astrocytes are heterogeneous in different CNS regions. Moreover, astrocytes are capable of assuming different morphologies and influence differential endothelial cell expression of tight-junction proteins that are critical for barrier formation between endothelial cells. For example, the blood spinal cord barrier is thought to be more permeable than the BBB because of the low number of pericytes and the reduced expression of tight- and gap-junction proteins (100). Therefore, structure and function of the neurovascular unit may be differentially affected by PAE in various CNS regions reflecting differences in PAE-induced cytokine responses (9, 45).

Peripheral Immune System Dysregulation

A strong and dynamic interplay exists between the CNS and the peripheral immune system (37). Specifically, during disease states, altered BBB permeability can provide improved access for circulating peripheral leukocytes that are able to directly interact with glia and neurons (36, 101, 102). Therefore, PAE-induced alterations of the peripheral immune system can be a strong modulator of CNS-immune interactions.

Clinically, fetal alcohol effects on peripheral immune competence have been suspected for a long time and multiple experimental studies support the possibility that PAE leads to long-term adverse effects on peripheral immune function (23, 103, 104). Dysregulation of cell-mediated immune responses have been reported with diminished T cell-proliferative responses (103, 105) and reduced antigen specificity of T cells (106). PAE has been associated with increased severity in influenza virus infection (106) and autoimmune arthritis (105) and a susceptibility to developing prostate cancer (107).

Collective evidence from other recent studies suggests that the PAE-related alteration of peripheral immune responses goes beyond T cells (52, 53). Published reports from our laboratory indicate that peripheral immune cells, especially myeloid cells ($CD11b^+$), seem to be partially activated during young adulthood with moderate PAE, as indicated by increased MHC2 and adhesion molecule expression along with increased cytokine (CCL2) production following *ex vivo* stimulation of splenic leukocytes (52). An extension of this initial work demonstrated that PAE-induced peripheral immune cell activation persists until late adulthood (53). While no significant change was observed in the overall PAE-derived T and B cell numbers compared with controls, basal increases in natural killer cells, and myeloid leukocytes were observed in secondary lymphoid organs. These results indicate possible basal activation of myeloid and natural killer cells in older animals despite the absence of immune challenge. In the same report, peripheral leukocytes (splenocytes and peritoneal leukocytes) from PAE rats displayed exaggerated expression of proinflammatory cytokines such as TNF and IL-1β following *in vitro* immune stimulation (53). Similarly, splenocytes from PAE offspring revealed augmented IL-1β production following *in vivo* LPS treatment (12).

During pathological conditions, a slightly different profile emerges. In the presence of nerve injury, PAE augments peripheral immune cell-derived proinflammatory cytokines IL-1β, TNF, and IL-6 at the site of nerve injury with a concurrent deficiency in IL-10 production (**Figure 1A**) (52). This PAE-induced exaggerated proinflammatory cytokine production could be due to reduced anti-inflammatory activity from multiple cellular sources and/or dysregulation of required interactions that occur among different immune cell subsets. For example, increased IL-1β and other proinflammatory cytokines produced by innate immune cells can bias increased differentiation of proinflammatory T cells (such as Th17 cells) (108, 109) and decrease immune inhibitory T regulatory (Treg) cells (110). Therefore, while speculative, it is possible that following subsequent inflammation during adulthood, PAE leads to a shift in T cell responses that are biased toward Th17- or Th1-like phenotypes and less toward a Treg phenotype. A possible Th17 bias may underlie a PAE-induced propensity to mount a proinflammatory response. Together, these studies indicate that peripheral immune cells are primed by low to moderate PAE leading to aberrant peripheral immune responses, which may underlie a susceptibility to developing autoimmune disease and inflammatory conditions following immune challenge in adulthood. If true, PAE produces a lifelong vulnerability to develop chronic immunopathological conditions.

PAE and Glutamate-Mediated Excitotoxicity

Glutamate, one of the best-studied excitatory neurotransmitters, plays a central role in the complex communication networks between neurons and glial cells. Glial glutamate transporters are crucial to ensure glutamate uptake after synaptic release in order to maintain glutamate homeostasis and avoid excessive neuronal excitation. Evidence exists that glutamate aspartate transporter

(GLAST), an astrocyte-specific glutamate transporter, is dysregulated under neuroinflammatory conditions (111). Alterations of glutamate reuptake and loss of glutamate transporters are associated with the presence of activated microglia and endangered neurons. Furthermore, proinflammatory mediators such as TNF (produced from glial and peripheral immune cells) can downregulate GLAST leading to impaired glutamate uptake activity (112). Additionally, a number of reports show aberrant glutamate transporter function is strongly linked with neurological disorders. For example, spinal glutamate transporter inhibition results in pathological pain thought to be caused by reduced glutamate clearance and enhanced glutamate-mediated excitation of spinal pain projection neurons (113). Similarly, in amyotrophic lateral sclerosis and multiple sclerosis, dysregulation of glutamate uptake is thought of as a potential mechanism of inflammation in the CNS (112, 114). It is notable that moderate pre- and postnatal alcohol exposure (throughout gestation until weaning at PND22) decreased the expression of GLAST (115). A decrease in glutamate uptake was also observed in hippocampal slices of these adolescent rats (115). Hence, a growing body of evidence indicates that PAE alters glutamate release and clearance by glial cells leading to excessive synaptic and peri-synaptic glutamate (**Figure 2**). Together, these events may contribute to an excessive neuroinflammatory microenvironment.

SUMMARY AND CONCLUSION OF CURRENT FINDINGS

To summarize, it is evident that PAE poses long-term consequences for neuroimmune function by reprogramming immune activity in the CNS and in the periphery. Therefore, moderate PAE may be a risk factor for various neurological diseases that involve immune-neuroimmune interactions. Though multiple studies report altered neuroimmune responses due to moderate PAE, most of these studies restricted their examination to a microglial role in producing inflammatory cytokines in the CNS. However, endothelial cells and astrocytes also produce and express receptors for many of the same cytokines and have been shown to be important in CNS-immune responses. Currently, a limited amount data exist identifying cell-specific contributions of PAE-related neuroinflammation. A better understanding of immune cell and astrocyte-specific roles may delineate PAE-related mechanisms underlying chronic CNS disease throughout the life span.

While vast majority of initial research in FASD has been conducted to explore the mechanisms of alcohol-induced neurotoxicity due to binge or high exposure of alcohol, in recent years, diverse effects of *moderate* PAE on the immune and neuroimmune systems have drawn significant attention. This area demands more extensive research given the fact that a significant percentage of the human population diagnosed with FASD does not readily display overt early-life indicators of PAE. Yet, these individuals remain vulnerable to impaired CNS function during childhood and likely throughout adulthood. Our current knowledge is based on, a significant amount of research on PAE and neuroimmune interactions driven by previous findings from heavy alcohol exposure (*in utero* and chronic alcoholism in adults). Therefore, extensive research using animal models, as well as careful dissection of existing data from high versus low PAE-induced effects on the developing and adult neuroimmune system will significantly enrich the field. Such work will aid in the identification of key neuroimmune factors underlying FASD, with the possible development of appropriate interventions that could significantly improve the quality of life for these individuals.

AUTHOR CONTRIBUTIONS

SN and EM prepared the manuscript and figures, provided substantial contribution to the intellectual content of the manuscript and figures.

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REVIEW
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Neuroimmunology of Behavioral Comorbidities Associated With Cancer and Cancer Treatments

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Behavioral comorbidities (depression, anxiety, fatigue, cognitive disturbances, and neuropathic pain) are prevalent in cancer patients and survivors. These mental and neurological health issues reduce quality-of-life, which is a significant societal concern given the increasing rates of long-term survival after various cancers. Hypothesized causes of behavioral comorbidities with cancer include tumor biology, stress associated with the cancer experience, and cancer treatments. A relatively recent leading mechanism by which these causes contribute to changes in neurobiology that underlie behavior is inflammation. Indeed, both basic and clinical research indicates that peripheral inflammation leads to central inflammation and behavioral changes in other illness contexts. Given the limitations of assessing neuroimmunology in clinical populations, this review primarily synthesizes evidence of neuroimmune and neuroinflammatory changes due to two components of cancer (tumor biology and cancer treatments) that are associated with altered affective-like or cognitive behaviors in rodents. Specifically, alterations in microglia, neuroinflammation, and immune trafficking to the brain are compiled in models of tumors, chemotherapy, and/or radiation. Evidence-based neuronal mechanisms by which these neuroimmune changes may lead to changes in behavior are proposed. Finally, converging evidence in clinical cancer populations is discussed.

Keywords: depression, cognition, cytokines, neuroinflammation, neuropathic pain

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INTRODUCTION

Over the past decade, advances in cancer diagnosis and therapy have increased the number of cancer survivors, substantially improving the relative percentages of 5-year survivors for the most common types of cancer in the United States (1). Regardless, cognitive impairments, fatigue, psychiatric comorbidities, and peripheral neuropathy, attributed largely to neurotoxic effects of cancer therapy, remain highly prevalent among cancer patients and survivors (2, 3). The cancer-related cognitive impairments are well-recognized and commonly referred to as "chemofog" or "chemobrain" [reviewed in Ref. (4)]. Indeed, intensity or duration of chemotherapy relates to the severity of chemobrain (5, 6), whereas psychological factors (e.g., depression) and surgery are largely independent (7). The cognitive domains most commonly implicated include learning and memory, concentration, executive function, and processing speed (8, 9), while the common psychiatric comorbidities include anxiety and depression (10). Cancer-related fatigue is characterized by persistent physical and mental tiredness that are not explained by recent activity and interfere with functional abilities

[reviewed by Bower (11)]. Chemotherapy-induced peripheral neuropathy (CIPN) is another maladaptive and debilitating side effect of cancer treatment consisting of allodynia, hyperalgesia, and neuropathic pain, observed in 30–68% of patients and persisting even after completion of chemotherapy (12). Of note, fatigue strongly correlates more with pain than with cognitive impairments or mood in patients with cancer (13–15). Together, these behavioral symptoms are debilitating and reduce quality-of-life by limiting functional independence, reducing adherence to cancer treatment, undermining social and professional life, and generating a high psychosocial stress burden (16–18). They can manifest acutely or chronically, persisting in 35–75% of cancer patients for months or even years after they are cancer-free (19–21). Such large discrepancies in prevalence are likely related to differences in cancer types and treatments or methodological assessments across studies. However, the biological mechanisms underlying these comorbidities remain unclear. Therefore, preventative approaches for behavioral changes have not been standardized and effective treatment remains a serious clinical problem (22). Substantial evidence has associated cancer treatment, especially chemotherapy, with brain damage and these behavioral comorbidities. The mechanisms by which chemotherapy induces brain neurotoxicity are hypothesized to involve neuroinflammation, damage associated molecular patterns (DAMPs) (23), impaired neurogenesis (24–32), oxidative stress, myelin degradation, and blood–brain barrier (BBB) degradation (33). Similarly, CIPN involves peripheral neuron damage and axonal degeneration (34, 35) *via* neuroinflammatory mechanisms in the spinal cord (34, 36–42).

Radiation therapy directed at the brain also has obvious effects on behavior and neuroimmunology (43, 44), whereas radiation directed outside of the brain was long considered localized and, therefore, without consequences on the brain. However, recent evidence in various cancer populations indicates that radiation directed away from the brain still induces fatigue, as well as executive function and memory problems that persist for years after therapy (45–47), potentially through the actions of radiation-induced bystander effects (e.g., inflammation).

In addition to cancer treatments, numerous studies demonstrate that tumor biology by itself is able to influence neurocognitive function and affect. For example, behavioral impairments are observed in treatment-free, tumor-bearing mice (30, 48–56) and in cancer patients before they start chemotherapy (57–69). Tumorigenesis is a complex and multistep process, consisting of tumor initiation, progression, and dissemination. The solid tumor microenvironment contains various non-tumor cell populations such as endothelial, stromal, and innate inflammatory immune cells that support tumor progression (70). Thus, peripheral-to-central inflammation has been implicated as a key pathway underlying these tumor-induced changes in behavior. In addition, tumors can affect endocrine stress pathways, thereby indirectly modulating neuroimmunology and behavior [reviewed by Pyter (71)]. This review will focus on the recent and expanding primary literature supporting a role for innate immunity and inflammation in tumor- and cancer treatment-induced behavioral symptoms.

Indeed, innate immune cell activation within the central nervous system (CNS) is a key factor driving neuroinflammation, with resident microglial cells as the primary cellular venue (72). Pattern recognition receptors, such as toll-like receptors (TLRs) and NOD-like receptors (NLRs), are constitutively expressed by microglia, astrocytes, and oligodendrocytes in the brain. These receptors recognize pathogen associated molecular patterns and DAMPs, which are "sterile" inflammatory signals released by dying cells in the periphery or brain (73). TLR activation elicits canonical NF-κB signaling, whereas NLR activation induces the assembly and activation of inflammasomes (multiprotein cytosolic complexes), each of which trigger pro-inflammatory caspases to cleave the pro-inflammatory cytokines, interleukin (IL)-1β, IL-18, and IL-33, into their active forms (74). Mounting evidence implicates microglial activation and its associated neuroinflammation in the pathogenesis of multiple neurological and psychiatric disorders, such as depression, Alzheimer's disease, multiple sclerosis, cognitive impairments, and normal aging (75–81). In terms of these chronic peripheral inflammatory conditions, basic science data indicate that cytokines can stimulate peripheral nerves (e.g., vagus) and/or humorally transduce inflammatory signals into the CNS and drive behavioral changes (82). In addition, recent studies indicate that TBI, stroke, and experimental autoimmune encephalomyelitis (multiple sclerosis model) increase BBB permeability (83–85), allowing inflammatory mediators and peripheral immune cells to directly enter the brain. Thus, it is possible that tumors or cancer treatments may also influence brain function by altering innate immune cell trafficking directly to the brain.

The pathway between cancer and the CNS is hypothesized to be bidirectional. Indeed, the concept that depression or stress may precipitate chronic inflammatory diseases, including cancer (86), has existed for centuries and has been reviewed elsewhere (87). Here, we focus on one direction of this bidirectional relationship: the tumor- and tumor treatment-induced neuroinflammation contributing to affective-like, pain, and cognitive behaviors. Cancer-related fatigue and its underlying immune mechanisms are thoroughly reviewed elsewhere (11). Understanding how tumor biology and cancer treatments can interact to lead to changes in the brain will allow for improved targeting by therapeutic interventions focused on behavioral issues and thereby increase quality-of-life and survival for cancer patients. Although behavioral comorbidities are relevant for both brain and peripheral tumor patients, brain tumors and their treatments impact the brain much more directly than peripheral tumors. Indeed, brain tumor effects on behavior are confounded by the fact that they physically disrupt the brain/brain immune system and their treatments directly target brain tissue; therefore, only tumors outside of the brain will be discussed here. It is important to note that despite relatively consistent behavioral issues reported among some cancer patients, their tumors, and cancer treatments are heterogeneous and complex. Finally, we focus on the most common cancer treatments of chemotherapy and radiation, however, cancer patients are also treated with other anticancer (e.g., immunotherapy), anti-nausea, anti-infection drugs, which likely further contribute to mental health issues.

RODENT MODELS OF CANCER, NEUROIMMUNOLOGY AND BEHAVIOR

Current basic research using rodent cancer models implicates several putative mechanisms underlying behavioral changes. These non-human models allow for a more neurobiological understanding of the effects of tumors and tumor treatments on behavior compared to clinical research. They can also elucidate the effects of specific cancer therapies by themselves by using tumor-free mice, thereby simplifying the complex interactions between tumors and multiple tumor treatments inherent to clinical populations.

The methods for identifying current reports in the English language on how cancer and cancer treatments drive behavioral and/or neuroimmune changes in rodent models consisted of PubMed searches through April 2018 using combinations of the MeSH search terms: ("rodent"; "cancer" or "neoplasms, experimental," or "tumor"; "inflammation" or "cytokine" or "microglia" or "neuroinflammation"; "behavior" or "cognition" or "learning" or "affect" or "depression" or "anxiety"; "chemotherapy" or "chemobrain" or "radiation" or "neuropathy" or "neuropathic pain"). Notably, only tumor models consisting of tumors located outside of the brain were considered. Here, we present tumor-bearing models with and without cancer treatments, followed by tumor-free models with cancer treatments.

Neuroimmunology in Tumor-Bearing Rodent Models

In solid peripheral neoplasms, tumor and non-tumor cells in the tumor microenvironment (e.g., leukocytes, fibroblasts, endothelial cells) secrete inflammatory mediators that attract additional immune cells, and promote tumor growth, development, and metastasis (70, 88, 89). Among the most common inflammatory mediators increased by tumors are cytokines and chemokines, including IL-1, TNF-α, IL-6, IL-8, IFN-α, IL-10, IL-12, TGF-β, and CXCR4 (90, 91). These inflammatory mediators are released into circulation and can be transduced into the brain potentially *via* neural and humoral pathways (92) leading to neuroinflammation, which in turn influences behavior (89) (**Figure 1**). Of note, increases in circulating cytokines are detectable only in some tumor models and during specific stages of the tumor development (89), although these humoral elevations are not mandatory to induce neuroinflammation and behavioral alterations (82).

Our previous review focuses on the behavioral consequences of tumors in rodents without cancer treatments (89). Several of these behavioral studies also report concomitant tumor-induced immune changes in the brain and/or in the periphery. For example, brain pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α), as well as inflammatory enzymes and signaling factors [nitric oxide synthase (iNOS), indolamine 2,3-deoxygenase, cyclooxygenase-2 (COX-2)], increase along with affective-like behavior, fatigue, or cognitive impairments, when various solid tumors are generated in the periphery [(30, 48, 50, 52, 54–56), but see Ref. (93)]. Likewise, circulating pro-inflammatory cytokine increases are frequently observed in solid tumor models [(30, 51, 53–55, 93, 94), but see Ref. (48, 51)]. These inflammatory changes are hypothesized to drive the accompanying behavioral changes, however, rarely are statistical relationships between the two assessed. Of the reports statistically linking behavior and inflammation, our lab and others demonstrate that circulating cytokines (51), tumor mass, and/or tumor-derived cytokine gene expression are positively associated with neuroinflammation, fatigue, or anxiety-like behavior in female mice with peripheral tumors (94). Indeed, the consistent increase in brain IL-1β levels in tumor-bearing mice (50, 55, 56, 95) suggests a putative role for inflammasomes not only during chemotherapy, but also in cancer-induced depressive-like behavior. However, a different mammary tumor model reports neuroinflammation, but in the absence of affective-like behavior, cognitive deficits, or changes in neurogenesis (93). These discrepancies in behavioral and neurobiological outcomes might be due to differing methodological approaches. While microglial activation is well-established in brain tumor models (96), recent evidence indicates that brain microglia may be the cellular source of this neuroinflammation in various peripheral tumor models, as is observed through increased ionized calcium-binding adaptor molecule 1 (Iba1) immunoreactivity or *Cd11b* gene expression in the cortex and hippocampus (brain regions that regulate affect, energy, and cognition) (55, 56, 94, 95) (**Table 1**). Similarly, microglial activation in the spinal cord is associated with bone pain in bone cancer models (34).

Furthermore, elevations in *Cd11b* and other neuroinflammatory mediators, as well depressive-like and sickness behaviors, are attenuated by minocycline anti-inflammatory treatment in murine models of colon cancer and human papilloma virus (HPV)-related neck and head cancer (55, 56). As further evidence that tumors appear to be causal, and perhaps have long-lasting consequences on microglial-related changes, complete surgical resection of non-metastatic mammary tumors partially reverses tumor-induced neuroinflammation and circulating cytokines, but amplifies anxiety-like behavior (94).

Although hippocampal microglial activation (*Cd11b* expression) at rest is consistent among the majority of these tumor models, the results concerning regional expression of *Cd11b* mRNA in the brain to a subsequent peripheral immune challenge are mixed. One such challenge, lipopolysaccharide (LPS) injection (i.p.) increases cortical and hippocampal *Cd11b* expression, but decreases its expression in the hypothalamus of mammary tumor-bearing rats (95). Moreover, in HPV-related neck and head tumor-bearing mice, LPS does not change hippocampal and cortical *Cd11b* expression (56). Such discrepancies in the neuroinflammatory response are likely related to differences in cancer types and LPS doses. Taken together, these results indicate that baseline inflammation and neuroinflammatory responses to secondary immune challenges are influenced by tumors, although the identification of specific underlying mechanisms requires further investigation.

While microglial cells are the primary drivers of neuroinflammation within the CNS, increasing evidence suggests that other neuroimmune mechanisms are associated with behavioral changes. For example, psychological stress induces myeloid-derived cell trafficking to the brain, which in turn, induces affective-like behavior (81). While this review focuses on

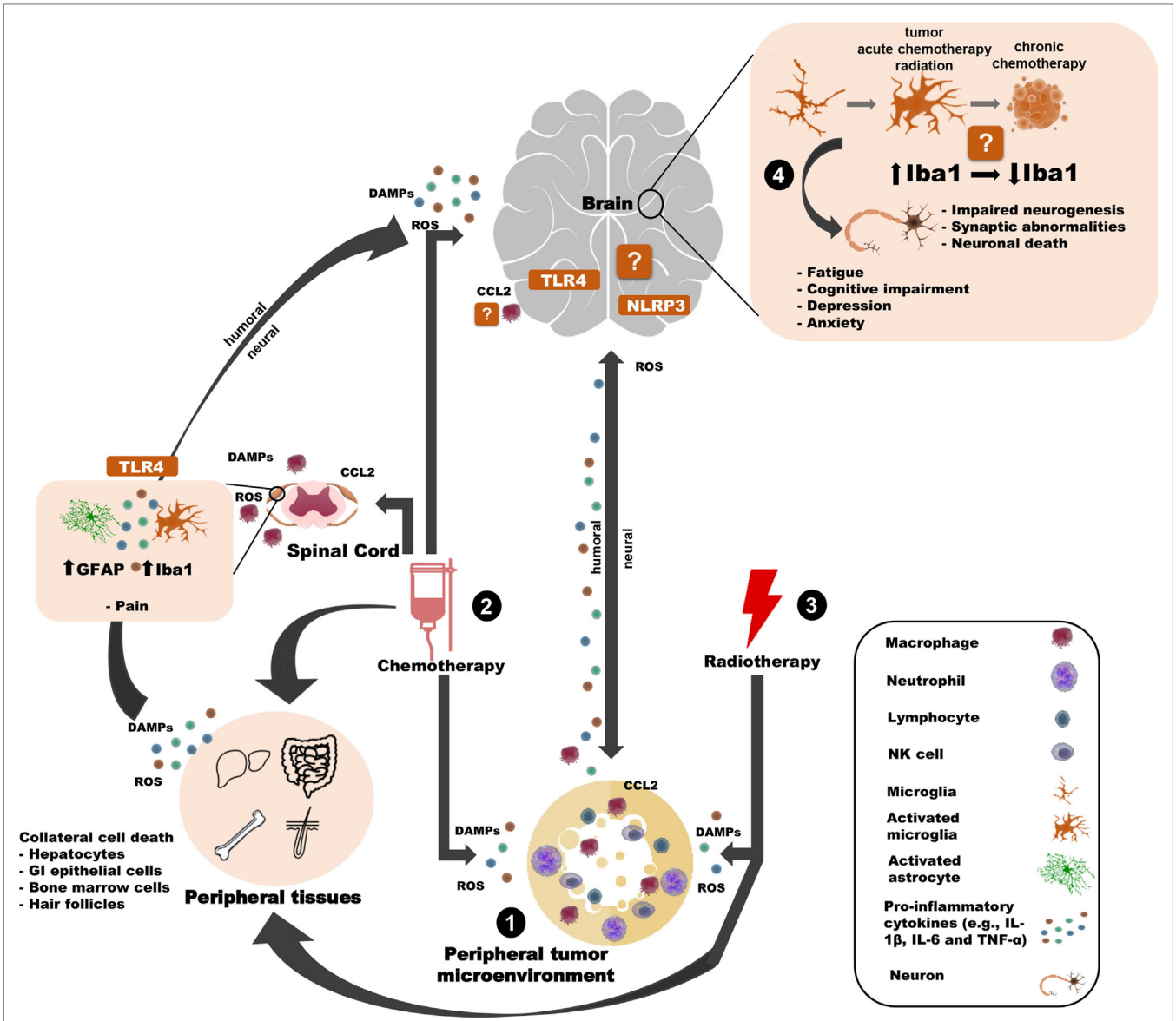

FIGURE 1 | Potential innate immune mechanisms by which peripheral cancer and cancer treatments can induce behavioral changes. (1) The tumor microenvironment releases pro-inflammatory mediators (e.g, cytokines) that can influence the brain and behavior through humoral or neural routes. (2) Chemotherapy induces cell death of tumor cells and healthy cells (in the brain and the periphery), thereby causing the release of DAMPs, ROS, cytokines, and chemokines and contributing to many side effects. For example, chemotherapy-induced peripheral neuropathy is associated with astroglial and microglial activation in the spinal cord and TLR4 activation in DRG neurons. Similar inflammasome activity may occur in the brain. Chemotherapy may also weaken the blood–brain barrier, allowing peripheral immune cells to traffic into/closer to the brain. (3) Peripheral radiotherapy induces cell death of tumor cells and healthy "bystander" cells and (indirectly) contributes to microglial activation and behavioral deficits. (4) Together, the tumor and cancer treatments influence microglia. Tumors and radiotherapy (indirectly) activate microglia, whereas chemotherapy may affect microglia differently over time. Microglia interface with neurons to affect behavior, potentially through. Certain elements of this work were taken and then adapted from somersault18:24 (Library of Science & Medical Illustrations). To view their site, visit http://www.somersault1824.com/. They are licensed under the Creative Commons Attribution-NonCommercial-ShareAlike 4.0 International License. To view a copy of this license, visit http://creativecommons.org/licenses/by-nc-sa/4.0/ or send a letter to Creative Commons, PO Box 1866, Mountain View, CA 94042, USA.

cancer and cancer treatments, it is important to note that stress associated with a cancer diagnosis may exacerbate tumor inflammatory activation, increasing tumor burden and development of metastases (97–100), and leading to more severe behavioral symptoms (51, 101, 102) through similar neuroimmune mechanisms. Furthermore, bone marrow-derived monocytes, including perivascular cells, meningeal macrophages, dendritic cells, and monocytes, have been implicated in the brain innate response in several neurologic and psychiatric diseases, as well as peripheral acute infections with sickness behavior (103–105). Indeed, in addition to potential humoral and neural routes by which peripheral inflammation is transduced into neuroinflammation, tumors

TABLE 1 | Summary of cancer- and cancer treatment-induced neuroinflammatory changes in rodents.

Reference	Treatment	Tumor	Behavioral effects	Central inflammatory measures
Pyter et al. (48)	No treatment	Rat mammary tumor	Depressive- and anxiety-like behaviors	↑ IL-1β, IL-6, TNF-α, and IL-10 (Hi)
Pyter et al. (50)	No treatment	Rat mammary tumor	Cognitive impairment	↑ IL-1β (Hi)
Pyter et al. (95)	No treatment	Rat mammary tumor	Impaired recovery from sickness behavior	↑ CD11b and IL-1β (Hi—basal conditions)
				↑ CD11b (Hi and Ctx—4 h after immune challenge)
				↑ IL-1β (Hi and Ctx—4 h after immune challenge)
Lebeña et al. (54)	No treatment	Mouse melanoma	Depressive-like behavior	↑ IL-6 and TNF-α (Hi)
Yang et al. (30)	No treatment	Mouse colorectal	Depressive-like behavior and cognitive impairment	↑ IL-6 and TNF-α (Hi)
				↓ COX-2 (Hi)
Norden et al. (55)	No treatment	Mouse colorectal	Depressive-like behavior (anhedonia)	↑ Microglia activation (Iba1$^+$ cells—Ctx)
				↑ IL-1β (Hi and Ctx) and IL-6 (Ctx)
Norden et al. (226)	No treatment	Mouse colorectal	Depressive- and fatigue-like behavior (prevented by ibuprofen)	↑ IL-1β and IL-6 (Hi; reduced by ibuprofen)
Walker et al. (93)	No treatment	Mouse metastatic mammary tumor	N/C	↑ IL-1β (Hi and Ctx)
		Mouse non-metastatic mammary tumor		N/C
Vichaya et al. (56)	No treatment	Mouse human papilloma virus-related neck and head cancer	N/C	↑ CD11b and TNF-α (Hyp—basal conditions)
				↑ IL-1β (Hi, Hyp, Ctx, CS, and Cb)
			Impaired motivated behavior, locomotor activity and depressive-like behavior 24 h after immune challenge	↑ IL-6 (Cb—24 h after immune challenge)
Pyter et al. (94)	No treatment	Mouse mammary tumor	Anxiety-like behavior	↑ CD11b and CXCL1 (Hi)
		Mouse mammary tumor resected	Tumor resection exacerbated anxiety-like behavior	Tumor resection reversed hippocampal CD11b and CXCL1 increase and ↑ cortical CXCL1
Seigers et al. (118)	Methotrexate	Tumor-free rat	N/A	↑ Microglia activation (Iba1$^+$ cells—Hi) and N/C in cytokines (Hi) or [11C]PK11195 uptake
Seigers et al. (113)	Cyclophosphamide	Tumor-free mouse	N/A	↓ Microglia (Iba1$^+$ cells—Ctx)
	Docetaxel			↓ Microglia (Iba1$^+$ cells—Ctx)
	Doxorubicin			N/C
	5-fluorouracil			↓ Microglia (Iba1$^+$ cells—Ctx)
	Methotrexate			N/C
	Topotecan			↓ Microglia (Iba1$^+$ cells—Ctx)
Christie et al. (29)	Cyclophosphamide	Athymic tumor-free nude rat	Cognitive impairment	↑ Microglia activation (ED1$^+$ cells—Hi)
	Doxorubicin			N/C
Paquet et al. (112)	Paclitaxel	Breast cancer xenograft in nude mouse	N/A	N/C
	Epirubicin + Cyclophosphamide	Breast cancer xenograft in nude mouse		↓ Microglia (Iba1$^+$ cells—Hi, CS, Ctx, and Cb) N/C
	Paclitaxel	Tumor-free nude mouse		N/C
	Epirubicin + Cyclophosphamide	Tumor-free nude mouse		↓ Microglia (Iba1$^+$ cells load—Hi, CS, Ctx, and Cb)
Yang, et al. (52)	Methotrexate	Mouse mammary carcinoma	Cognitive impairment and depressive- like behavior	↑ iNOS and COX-2 (Hi)
				↑ Microglia (Iba1—Hi)
Salas-Ramirez et al. (117)	Doxorubicin + Cyclophosphamide	Tumor-free rat	Cognitive impairment	↑ Erk1/2 and Akt activation in OVX female rats (Hi)
Zhang et al. (129)	Paclitaxel	Tumor-free rat	Neuropathic pain	↑ Astrocytes activation (GFAP$^+$ cells in the spinal cord)
Zhang et al. (38)	Paclitaxel	Tumor-free rat	Neuropathic pain (attenuated by anti-CCL2 treatment)	↑ CCL2 (spinal astrocytes)

(*Continued*)

TABLE 1 | Continued

Reference	Treatment	Tumor	Behavioral effects	Central inflammatory measures
Pevida et al. (40)	Paclitaxel	Tumor-free mouse	Neuropathic pain (prevented by anti- CCL2 or minocycline treatment)	↑ CCL2 (lumbar spinal cord) ↑ Microglia (Iba1$^+$ cells in the lumbar spinal cord)
Ruiz-Medina et al. (126)	Paclitaxel	Tumor-free mouse	Neuropathic pain	↑ Microglia and astrocytes (Iba1$^+$ and GFAP$^+$ cells in the spinal cord)
Mannelli et al. (128)	Oxaliplatin	Tumor-free rat	Neuropathic pain (prevented by pharmacological microglia or astrocyte inhibition)	↑ Microglia and astrocytes activation (Iba1$^+$ and GFAP$^+$ cells in the dorsal horn)
Huang et al. (39)	Paclitaxel	Tumor-free rat	Neuropathic pain (attenuated by anti-CX3CL1 treatment)	↑ CX3CL1 and caspase-3 (A-fiber primary sensory neurons) ↑ Macrophages infiltration (DRG; prevented by anti-CX3CL1 treatment)
Li et al. (37)	Paclitaxel	Tumor-free rat	Neuropathic pain (transiently reversed by TLR4 antagonist treatment)	↑ TLR4 (spinal astrocytes and DRG neurons), MyD88, and TRIF (DRG neurons)
Li et al. (36)	Paclitaxel	Tumor-free rat	Neuropathic pain (prevented by MAPK inhibitors)	↑ pERK1/2 and pP38 (DRG) ↑ TLR4 signaling *via* MAP kinases and NF-κB (DRG)
Zhang et al. (42)	Paclitaxel	Tumor-free rat	Neuropathic pain (reduced by intrathecal TLR4 antagonist treatment, CCL2 neutralization or macrophage depletion)	↑ TLR4 activation, CCL2 expression, and macrophages infiltration (DRG—reduced by intrathecal TLR4 antagonist treatment or CCL2 neutralization)
Makker et al. (41)	Oxaliplatin Paclitaxel	Tumor-free mouse	Neuropathic pain	↓ Microglia (P2ry12$^+$ cells—dorsal/ventral horns) ↓ Microglia (P2ry12$^+$ cells—dorsal/ventral horns) ↑ TNF-α, IFN-γ, CCL11, CCL4, CCL3, IL-12p70, and GM-CSF (spinal cord)
Jia et al. (147)	Paclitaxel	Tumor-free rat	Neuropathic pain (alleviated by a non-specific ROS scavenger)	↑ NLRP3, caspase-1, and IL-1β (DRG) reversed by a non-specific ROS scavenger ↑ NLRP3 in CD68$^+$ macrophages and (DRG and sciatic nerve) Mitochondrial damage (spinal cord)
Ledeboer et al. (127)	Paclitaxel	Tumor-free rat	Neuropathic pain (attenuated by intrathecal IL-1 receptor antagonist or IL-10 gene therapy)	↑ Microglia activation (OX-42 and OX-6$^+$ cells in the spinal cord) ↑ CD11b, TNF-α, and IL-1β (DRG), attenuated by intrathecal IL-10 gene therapy
Hu et al. (227)	Cisplatin	Tumor-free mice	Neuropathic pain (attenuated by minocycline or anti-TREM2 treatment)	↑ TREM-2-mediated microglia activation (Iba1$^+$ cells in the spinal cord) N/C in astrocyte activation ↑ IL-6, TNF-α, IL-1β, iNOS, and CD16 (spinal dorsal horn—attenuated by intrathecal minocycline) and TREM-2 (spinal cord)
McGinnis et al. (9)	Radiotherapy + Anti-CTLA-4	Tumor-free BALB/c mouse Tumor-free C57BL/6J mouse BALB/c mouse colorectal C57BL/6J mouse lung carcinoma	↓ Anxiety-like behavior (in some cases) and ↑ Cognitive impairment (in all cases)	↑ Microglia activation (CD68$^+$ cells—Ctx and Hi)
Feiock et al. (21)	Radiotherapy Methotrexate	Tumor-free mouse	N/A	↑ Microglia (Iba1$^+$ cells—Hi, CS, Ctx, and Cb) and astrocyte (GFAP$^+$ cells—CS, Ctx, and Cb) activation and TNF-α (Hi)

(*Continued*)

TABLE 1 | Continued

Reference	Treatment	Tumor	Behavioral effects	Central inflammatory measures
Acharya et al. (123)	Cyclophosphamide	Athymic tumor-free nude rat	Cognitive impairment (ameliorated by stem cell transplantation treatment)	↑ Microglia activation (CD68$^+$ cells—Hi) reversed by stem cells transplantation treatment
Cheruku et al. (229)	Doxorubicin	Tumor-free rat	Cognitive impairment (ameliorated by Catechin treatment)	↑ MPO levels (Hi and Ctx) reversed by Catechin treatment
El-agamy et al. (230)	Doxorubicin	Tumor-free rat	Cognitive impairment ameliorated by Astaxanthin treatment	↑ TNF-α, PGE_2, and COX-2 levels (Hi) and astrocytes activation (GFAP$^+$ cells) reversed by astaxanthin treatment
Ramalingayya et al. (231)	Doxorubicin	Tumor-free rat	Cognitive impairment ameliorated by Rutin treatment	↑ TNF-α levels (Hi and Ctx) reversed by astaxanthin treatment

IL-1β, interleukin-1 beta; IL-6, interleukin-6; TNF-α, tumor necrosis factor alpha; IL-10, interleukin-10; Cd11b, cluster of differentiation molecule 11b; COX-2, cyclooxygenase-2; Iba1, ionized calcium-binding adaptor molecule 1; CXCL1, C-X-C motif chemokine ligand 1; [11C]PK11195, (1-(2-chlorophenyl)-N-methyl-N-(1-methylpropyl)-3-isoquinoline carboxamide); ED-1, anti-CD68; CD68, cluster of differentiation 68; iNOS, inducible nitric oxide synthase; ERK1/2, extracellular signal-regulated protein kinases 1 and 2; Akt, protein kinase B; OVX, ovariectomized; GFAP, glial fibrillary acidic protein; CCL2, C–C motif chemokine ligand 2; CX3CL1, C-X3-C motif chemokine ligand 1; TLR4, toll-like receptor 4; Myd88, myeloid differentiation primary response 88; TRIF, TIR-domain-containing adapter-inducing interferon-β; pERK1/2, phospho-extracellular signal-regulated protein kinases 1 and 2; pP38, phospho-P38; MAP, mitogen-activated protein kinase; NF-κB, nuclear factor-κB; P2ry12, purinergic receptor P2Y; INF-γ, interferon gamma; CCL11, C–C motif chemokine ligand 11; CCL4, C–C motif chemokine ligand 4; CCL3, C-C motif chemokine ligand 3; IL-12p70, interleukin-12p70; GM-CSF, granulocyte-macrophage colony-stimulating factor; NLRP3, nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3; OX-42, anti-complement type 3 receptors; OX-6, anti-major histocompatibility complex class II; TREM-2, triggering receptor expressed on myeloid cells 2; CD16, surface Fcγ receptor; MPO, myeloperoxidase; PGE2, Prostaglandin E2; Hi, hippocampus; Hyp, hypothalamus; CS, corpus striatum; Ctx, cortex; Cb, cerebellum; DRG, dorsal root ganglion; N/C, no change; N/A, not applicable.

affect immune trafficking to various areas of the body (94). Thus, immune trafficking of circulating monocytes to the brain may also play a role in tumor-induced changes in neurobiology and behavior (106–108) and warrants investigation.

Neuroimmunology and Cancer Treatments in Tumor-Bearing Rodents

Chemotherapy is a common adjuvant cancer treatment (109). While most basic science reports focus separately on either tumors or chemotherapy, a few combine the two for a more clinically-relevant (albeit complex) model. The combination of tumors and chemotherapy could additively increase peripheral inflammation or potential BBB disruption, thereby allowing peripheral inflammatory mediators to reach the brain, induce neurotoxicity and neuroinflammation, and contribute to cognitive and affective symptoms (8, 25, 52).

Both human and non-human research suggests that cancer treatment is causally related to the development of mood and anxiety disorders, although the potential underlying mechanisms remain broad. For example, antimetabolite chemotherapy (methotrexate) induces significant depressive-like behavior and cognitive impairments associated with an upregulation of pro-inflammatory enzymes (iNOS and COX-2) and activation of microglia in the brains of mammary tumor-bearing mice (52). In contrast, methotrexate suppresses peripheral cytokine levels in other studies (110, 111). Combined epirubicin and cyclophosphamide chemotherapies reduce stereological hippocampal microglial Iba-1 expression in the hippocampus, cortex, striatum, and cerebellum of both tumor-free and nude mice xenografted with patients' tumor samples compared to xenografted mice treated with saline (112). These Iba1 reductions may represent chemotherapy-induced microglial cell death (113). Behavioral changes were not assessed in this study. Taken together, the mixed inflammatory results from tumor-bearing models treated with chemotherapy indicate that tumor-free models are still necessary to clarify the individual roles of chemotherapeutic agents and tumors in associated behavioral impairments.

Emerging basic and clinical research indicates that stress and cancer treatments interact to influence tumor-associated immune and behavioral symptoms (101, 114). For example, physical restraint stress in tumor-bearing mice treated with cyclophosphamide impairs the antitumoral immune response, thereby reducing the therapeutic effects of the chemotherapy treatment (115). The potential synergistic effects of stress on cancer treatment-induced neuroinflammation remain to be determined. Finally, limited data are available on radiation as another potential cancer treatment contributor to neuroinflammation and/or behavioral consequences in tumor-bearing rodent models. One recent study evaluated these changes in tumor-free and tumor-bearing mice that received peripheral radiotherapy or immunotherapy (anti-CTLA-4 antibody) or both. Of note, the mice received precise peripheral irradiation to the tumor site in the right flank. Immunotherapy alone or in combination with radiotherapy induces cognitive impairments, increases in CD68$^+$ microglial immunostaining and central cytokine production (9). Thus, the current literature concerning neuroinflammatory-dependent behavioral changes in rodent cancer models indicates that a variety of cancer treatments are likely relevant, despite their different mechanisms of action.

Neuroimmunology and Cancer Treatments in Tumor-Free Rodent Models

The most extensive investigation regarding the potential mechanisms by which cancer treatments alter behavior is derived from studies using chemotherapeutic agents in tumor-free rodent models. Within this literature, some reports focus on behavioral

consequences, neuroimmune consequences, or both. Notably, the reported behavioral effects vary based upon the particular agents and administration paradigms used, as well as the specific behavioral tests employed. The short- and long-term behavioral changes following chemotherapy treatment predominantly consist of impaired performance in learning and memory tasks including reference and working spatial performance, novel object recognition, and object placement without affecting general motor function (113, 116, 117). The majority of these studies report generalized hippocampal and cortical cellular or myelin (protective sheath of neuronal axons) damage in the brain, with some evidence of microglial cell death (25, 29, 118–120). Biochemical testing of some chemotherapeutic agents indicates that they should not be able to cross the BBB (121), alternatively suggesting that chemotherapy metabolites or other indirect mechanisms, such as peripheral immune cell infiltration, may be driving these neurobiological consequences (25). How neuroinflammation can alter neuronal function to cause behavioral changes is discussed in section "Link Between Neuroimmunology and the Neuroscience of Behavior."

Several reports indicate a role for neuroimmune activation in chemotherapy-induced behavioral deficits (**Table 1**). For example, methotrexate induces microglia activation (Iba-1$^+$ staining) in the hippocampus 1 and 3 weeks after treatment in tumor-free rats, in addition to reducing hippocampal blood vessel density (122). However, the peripheral cytokine levels and positron emission tomography scans for the uptake of [11C]PK11195 (a marker that has been associated with neuroinflammation and increased microglia activation) do not support neuroinflammatory changes underlying to immunohistochemistry results. Similarly, clinically-relevant dosing of chronic cyclophosphamide and doxorubicin treatments in tumor-free rats induces impairments in hippocampal-based memory and reduces neurogenesis (29). Coincident with the behavioral and neurogenesis changes, cyclophosphamide, but not doxorubicin, induces microglia activation (ED-1$^+$ staining). In another report, impairments in different cognitive domains were observed in tumor-free mice after cyclophosphamide, docetaxel, doxorubicin, 5-fluorouracil, methotrexate, or topotecan treatment (116), while a reduced number of microglial cells (Iba-1$^+$ cells) were observed in the prefrontal cortex for all these treatments (three weeks after treatment) compared with control mice, except methotrexate and doxorubicin treatment (113). In addition, chronic cyclophosphamide treatment in athymic nude rats induces microglial activation (increased CD68$^+$ cells) in the hippocampus, as well as cognitive impairments in hippocampal and cortical-dependent tasks (123). These mixed microglial results indicate that multiple chemotherapeutic mechanisms of action may converge to trigger neuroinflammation and behavioral changes and that neuroinflammation may be due to microglial activation, microglial cell (or other cell) death or even disruptions in microglial homeostasis. Interestingly, some studies have reported a correlation between chemotherapy-induced circulating and central pro-inflammatory cytokine (IL-6, TNF-α, and IL-1β) concentration and behavioral changes acutely, but not chronically, suggesting that different mechanisms might be driving the initiation and the persistence of these comorbidities (124, 125).

Several chemotherapeutic drugs, such as platinum-based drugs (cisplatin, oxaliplatin), vinca alkaloids (vincristine), and taxanes (paclitaxel and docetaxel), trigger CIPN [as reviewed by Starobova and Vetter (35)] that is associated with neuroinflammation, although the literature is rather conflicting. Some studies indicate a key role for microglial activation [increased Iba-1, OX-42 (complement type 3 receptors), OX-6 (major histocompatibility complex class II) immunoreactivity, and *Cd11b* gene expression] in the spinal cord, or specifically, the dorsal root ganglion (DRG) of sensory neurons within the spinal cord (40, 126, 127). This microglial activation can be reversed by minocycline antibiotic treatment (40, 128) or intrathecal anti-inflammatory IL-10 gene therapy (127). However, the majority of CIPN studies implicate astrocyte activation (increased GFAP immunoreactivity and astrocyte hypertrophy) (36, 37, 41, 126, 128, 129). With CIPN, paracrine activation of CCL2/CCR2 signaling occurs and/or increased levels of CX3CL1 drive immune trafficking of activated macrophages to DRG, inducing nerve damage (39), which can be inhibited by anti-CCL2 antibody treatments or macrophage depletion (38–40, 42). Moreover, recent studies reported an increase in TLR4 signaling in spinal cord astrocytes and neurons in the DRG (36, 37). Potential pathways by which paclitaxel chemotherapy contributes to CIPN *via* TLR4 activation are the downstream canonical (myeloid differentiation primary response gene 88) and non-canonical pathways (TIR-domain-containing adapter-inducing interferon-β), culminating in NF-κB activation and upregulation of pro-inflammatory cytokines and chemokines including TNF-α, IFN-γ, IL-6, and CCL2 (36, 37, 41). TLR4 activation has been also associated with the sensitization of the ionic channel transient receptor potential vanilloid subtype 1 (TRPV1) (36, 130), found in nociceptors. Oxaliplatin chemotherapy also induces CIPN by upregulating pro-inflammatory cytokines and chemokines (IL1-β, TNF-α, IL-6, IL-8, and CCL2), which sensitizes nociceptors. The adaptive immune system is also likely involved in these responses as both paclitaxel and oxaliplatin increase the circulating levels of CD4$^+$ and CD8$^+$ T-cells (41). In addition, one study (131) indicates that the NLRP3 inflammasome pathway may also be activated *in vitro* in primed murine bone marrow-derived macrophage during anthracycline-induced IL-1β release. This suggests that some of the associated side effects, including behavioral changes, may be attenuated by IL-1β suppression. Indeed, intrathecal injection of IL-1ra transiently reversed paclitaxel-induced allodynia (127). NLRP3 inflammasome activation is driven by mitochondrial damage and reactive oxygen species (ROS) production in infiltrated macrophages of DRG and peripheral nerves and is also thought to play a role in paclitaxel-induced CIPN. Of note, some studies implicate neurotoxic effects of antineoplastic agents, which impair axonal trafficking leading to myelin and axon damage in CIPN, suggesting that the cellular damage may precede the neuroinflammation in the DRG [reviewed by Nicolini et al. (132)].

Finally, the induction of biological consequences on cells that are not directly transected by radiation treatment due to the signaling of those cells that are, is termed radiation-induced bystander effects. Both *in vitro* and *in vivo* models demonstrate off-target consequences of radiation on epigenetics, DNA health,

apoptosis, cell proliferation, tumorigenesis, and inflammation (133). Indeed, peripheral radiation treatment to the right hind limb in tumor-free mice increases microglial Iba1$^+$ cell numbers and TNF-α gene expression in the brain, comparable to the neuroinflammation observed following chemotherapy treatment (21). Whole-body radiation-induced neuroinflammation is associated with pro-inflammatory gene expression and reduced locomotion (134), although the direct brain radiation may be responsible for these effects. Radiation in rodents is also associated with general increases in circulating inflammation (135, 136), which coincide with fatigue (i.e., reduced locomotion) (137, 138). Despite the modest behavioral data currently available after radiation treatment, the reported peripheral and neuroinflammatory responses suggest that radiation can contribute to behavioral changes.

Other major gaps in understanding neurobiobehavioral changes in the context of cancer and cancer treatments pertain to the role of peripheral myeloid cells (monocytes, macrophages, and dendritic cells) and their potential localization to brain areas that interface with the peripheral circulation, such as the choroid plexus, perivascular spaces, and meninges. The chemokine CCL2 regulates myeloid cell infiltration (and potential inflammation) to different tissues, including these brain areas. For example, while CCL2 ablation in mice increases the peripheral pro-inflammatory cytokine response to LPS, it decreases the neuroinflammatory response in the entorhinal and frontal cortices and the hippocampus (139). Furthermore, CCL2 released by brain glioma tumors plays a key role in recruiting myeloid cells to the brain (140). However, the extent to which chemokine release by tumors in the periphery may influence immune cell trafficking to the brain and behavior remains unclear. On the other hand, CCL2 or peripheral immune cell trafficking to the brain may play a role in the context of chemotherapy-induced behavioral changes, as CCL2 ablation improves 5-FU chemotherapy-induced fatigue in tumor-free mice (141). A similar trafficking mechanism in spinal cord and DRG is also hypothesized to influence the development and persistence of CIPN (39, 42). The common neutropenia and lymphopenia side effects of chemotherapy may appear to conflict with the potential for increased innate immune cell trafficking to the brain and inflammation at first glance (142). When in fact, this immunogenic cell death results in production of DAMPs (proteins, nucleic acids, purines, and ROS), priming of CD4$^+$ and CD8$^+$ lymphocytes and a robust antigen-specific immune response against dead cell-associated antigens (143–145). Thus, cancer treatments overall consistently increase inflammation. These inflammatory mediators activate inflammasomes as well as TLRs to induce immunosurveillance or tumor progression (146), but also contribute to neuroinflammation, depression and neuropathic pain (147).

In summary, converging lines of evidence suggest that cancer and cancer treatments induce neuroinflammatory and behavioral changes in rodent models (**Figure 1**). Nevertheless, expansion of these initial basic science findings is required (**Table 1**). Specifically, the moderate variability in current microglial-related results from brain samples of models of tumors and cancer treatments necessitates a thorough temporal screening of brain microglial functioning and neuroinflammatory responses throughout tumor development and chemotherapy/radiotherapy treatments. This type of investigation would help to identify the cellular source/s of inflammation in the brain and elucidate the causal role of microglia in associated behavioral changes. Finally, several alternative pathways, including the sympathetic nervous system and the hypothalamic–pituitary–adrenal axis, modulate immune functions and, therefore, may be involved in the interaction among peripheral cancer, inflammation, and the brain.

Link Between Neuroimmunology and the Neuroscience of Behavior

Support for neuroimmune signaling that is associated with changes in behavior is extensively reviewed elsewhere in the context of peripheral tumors alone (89) or chemotherapy (20, 23, 148). As previously discussed, peripheral inflammation due to cancer or chemotherapy can trigger microglial activation and associated neuroinflammation. In some reports, this neuroinflammation is associated with changes in neurons. For example, chronic cyclophosphamide chemotherapy treatment induces cognitive impairment, microglial activation, and impaired neuronal architecture (123). Attenuation of this neuroinflammation reverses the neural and behavioral changes, suggesting that the neuroinflammation preceded the structural changes to the neurons. Alternatively, chemotherapy may damage brain tissue directly, heralding in the inevitable local neuroinflammatory response. Indeed, chemotherapy induces brain cell death (e.g., *via* ROS production), synaptic damage, DAMP production, disruption of the BBB, mitochondrial dysfunction, white matter damage, and alterations in neurotransmitter availability (149, 150). For example, reports from multiple labs indicate that antimetabolite chemotherapy (5-fluorouracil; 5-FU) crosses the BBB thereby directly reducing myelination and neurogenesis, as well as disrupting learning and memory (27, 151, 152). In addition, intrahippocampal human neural stem cell treatment reverses the hippocampal microglial activation and impaired neuronal architecture, as well as cognitive impairments induced by chronic cyclophosphamide treatment in athymic nude rats (123), which suggests that the neuronal damage caused microglial activation. Regardless of the order of the events, converging evidence indicates that different inflammatory microenvironments can drive various microglia phenotypes that interact with CD4$^+$ CD45$^+$ cells to induce neuroprotection, neurodestruction, or unchanged neurobiology (153). While these direct microglial–neuron interactions have not yet been demonstrated in the context of cancer, other examples are available. Chronic stress-induced depressive-like behavior is mediated by an initial phase of microglial activation and proliferation followed by microglial apoptosis and suppressed hippocampal neurogenesis (154). Indeed, this dynamic microglial pattern may be similar to that over early and late periods of time after chemotherapy treatment. Furthermore, although microglia are most well-recognized for innate immune functions, increasing data indicate that non-pathological microglial functions are essential for normal brain development, as well as structural and functional processes in the adult CNS (155). In the healthy brain, microglia regulate the development and plasticity of neuronal circuit architecture, modulate synapse development, activity, and elimination, as well as modulate neurogenesis (156, 157). Thus, microglial activation under inflammatory conditions (potentially cancer or cancer treatments) also likely interferes with these basic neurobiological functions and thereby alters behavior.

Alternatively, microglia may indirectly affect neuronal function *via* astrocytes. Microglial activation has been shown to induce ATP release, which in turn stimulates purinergic receptors on astrocytes to modulate nearby neuronal electrophysiology (158). Although this work is *in vitro*, the interaction between the various glial cells and neurons constitute another putative mechanism for cancer-associated behavioral changes and an interesting area for future studies. Furthermore, a recent study indicates that the serotonergic pathways downstream of the serotonin $(5\text{-HT})_{2B}$ receptor in microglial cells contribute to neuronal synaptic refinement and brain maturation (159). These same 5-HT receptors can inhibit TLRs, thereby counteracting inflammation (160), and may, therefore, be a potential target to prevent cancer-associated behavioral changes. Indeed, an increasing number of studies suggest an involvement of the serotonergic system in the modulation of innate and adaptive immune functions (160–162).

CANCER PATIENTS

Neuroimmunology and Cancer Treatments

For this section, the approach for finding original reports in the English language that considered neuroinflammatory factors and/or psychological and behavioral symptoms in cancer patients with tumors outside of the CNS before and after cancer treatment consisted of PubMed searches through April 2018 using combinations of the MeSH search terms: "depression," or "anxiety," or "cognition," or "neuropsychological test"; "cancer," or "tumor" or "chemotherapy" or "radiation"; "inflammation" or "imaging" or "microglia" or "brain."

In the field of cancer research, it is well-accepted that affective disorders and cognitive impairments are more highly prevalent in cancer patients before, during and even years after cancer treatment relative to non-cancer controls (10, 163, 164). Although, cancer and cancer treatments share some potentially confounding physical symptoms (cachexia, fatigue, sleep disturbances) with major depressive disorder (MDD), meta-analyses and systematic reviews indicate that affective (MDD and "depressive") and cognitive impairments are independent of these physical symptoms in cancer patients (165–168).

Although it has often been proposed that neuroinflammation may underlie the affective and cognitive deficits observed in cancer patients (23, 89, 169), scant neuroscientific data are attainable in patients. The most relevant clinical approach for understanding the relationship between neuroscience and cancer-associated behavioral comorbidities is neuroimaging. Of this neuroimaging work in cancer patients, studies focused on the effects of chemotherapy are most abundant, reviewed in Ref. (170). In the dominant breast cancer literature, cross-sectional neuroimaging approaches have yielded mostly consistent chemotherapy-induced deteriorations in neurostructure (using diffusion tensor imaging), some of which have been correlated with poor cognitive performance (171, 172). Specifically, cancer treatments reduce brain white and/or gray matter in the corpus callosum and cortex (173) or reduce hippocampal volume (171). These structural impairments are detectable over 20 years post-chemotherapy (174), and in fact, may be progressive (175, 176). Neuroinflammation is a top potential mechanism by which this occurs (177). Functional magnetic resonance imaging results (e.g., hippocampal activation during a cognitive task or at rest) are more mixed for chemotherapy-treated survivors (4, 178–181), perhaps due to the increased complexity of these assessments during active behavior. Neuroimaging cannot yet directly address the neuroinflammatory hypothesis; however, alterations in neuroimaging have been recently associated with peripheral inflammation in cancer patients treated with chemotherapy or radiation (182) and are associated with peripheral immune activation in other populations (183–186). Altered neuroimaging is also demonstrated in cancer patients prior to treatment, indicating that tumors outside of the brain influence brain network dynamics on their own (187, 188), possibly through immune signaling. For example, in breast cancer survivors at least 6 months after cancer treatment completion, peripheral inflammation is more strongly associated with amygdala reactivity to socially threatening images than in cancer-free controls (182).

Although less direct than neuroimaging, the positive association between cancer behavioral comorbidities and circulating inflammatory markers corroborates the neuroinflammatory theory and is well-supported (11, 58). In addition to baseline peripheral inflammatory markers, *in vitro* reactivity of peripheral immune cells is elevated in cancer patients with negative behavioral symptoms (189, 190), as are allelic profiles characterized by cytokine deregulation (191), and genetic polymorphisms of the inflammatory pathway [(192–194), but see Ref. (195)]. Furthermore, cytokine-based immunotherapy (IFN-α, IL-2 infusions) causes depression and cognitive impairments in cancer patients and other medically ill patients (196–198). Finally, there is a single neurobiological record of four adult (non-brain) cancer patients after high-dose chemotherapy treatment (199). Neuropathology is similar among these cancer patients and includes the loss of myelin and axons, as well as fluid and macrophage infiltration in various CNS regions. Similar brain pathology has been reported in autopsies of children with leukemia who were treated with chemotherapy (200). Taken together, these studies are consistent with the hypothesis that neuroimmune activation may be a key underlying mechanism of chemotherapy-induced cognitive impairment in cancer patients.

Many clinical studies that focus on chemotherapy effects on brain and behavior include cancer patients that also receive ionizing radiation therapy, although the individual role of radiation is rarely delineated. Thus, the effects of radiation therapy on neurobiology are poorly understood compared with chemotherapy. This oversight is relevant to many cancer patients; for example, radiation is used to treat approximately 56% of breast cancer patients (201). Abscopal effects, by which radiation used to treat a proximal tumor also reduces distal tumors, are thought to be immune-mediated (202). Specifically, dendritic cells and macrophages phagocytose cells damaged by radiation and then present tumor debris to adaptive immune cells to trigger widespread anti-tumor actions (203). As a result, circulating cytokines are elevated during radiation therapy in some cancer studies (204–206), but not others (207, 208). Breast cancer patients with higher baseline circulating inflammatory markers (C-reactive protein, myeloid-derived cells, IL-6) are also predisposed to fatigue after radiation (209). Thus, the potential for radiation-induced peripheral inflammation to potentiate neuroinflammation remains a viable hypothesis in need of further testing.

ANTI-INFLAMMATORY INTERVENTIONS (PHARMACOLOGICAL AND NON-PHARMACOLOGICAL) IN RODENTS AND HUMANS

To date, there are no standard clinical interventions for cancer behavioral comorbidities. Interventions used to reduce these behavioral issues by targeting inflammatory mechanisms include exercise, psychosocial interventions (210), and pharmacological anti-inflammatory treatments. Of note, pharmacological anti-inflammatory treatments have potential hematologic toxicity and cardiovascular side effects and may interact with other cancer treatments (211); therefore, greater emphasis has been placed on non-pharmacological interventions.

In breast cancer patients after chemotherapy, 12 weeks of hatha yoga improves self-reported cognitive function while reducing circulating inflammatory markers (212). Similar results were observed after 6-weeks of aerobic walking and resistance training (213) with the addition of increases in circulating anti-inflammatory markers. In the latter study, reductions in inflammation correlate with cognitive improvements. The same duration of lyengar yoga also reduces fatigue (214) while decreasing pro-inflammatory NF-κB activity (215). Furthermore, Qigong intervention (Chinese coordinated body posturing and movement) reduces circulating C-reactive protein as well as improves self-reported cognitive functioning (216).

In a subset of depressed cancer patients, 4 months of psychosocial intervention (relaxation and stress reduction exercises and education) improves mood while reducing inflammatory markers (217). Furthermore, cognitive–behavioral stress management intervention reduces pro-inflammatory gene expression of circulating immune cells from breast cancer patients, while decreasing negative affect and increasing positive affect relative to standard-of-care controls (218). However, other cognitive-based training that reduces depression and anxiety, increases inflammatory cytokine production in stimulated immune cells *in vitro* in breast cancer patients (219, 220). Finally, resistance-based exercise reduces radiation-induced increases in circulating pro-inflammatory cytokines, which mediates slight improvements in fatigue and pain (205). In another breast cancer subpopulation, characterized by mild to moderate depression and pain, a nonsteroidal anti-inflammatory drug that specifically inhibits COX-2 (celecoxib) improves depressive symptoms better than a non-selective COX inhibitor (211). Drugs that interfere with TNF-α signaling also improve fatigue in chemotherapy-treated cancer patients (221, 222).

In rodent models, similar interventions to reduce cancer treatment side effects include exercise and pharmacological anti-inflammatory treatments. Several studies indicate that voluntary (223) or forced (224, 225) aerobic exercise prevent cognitive impairments in chemotherapy-treated or brain-irradiated, tumor-free mice compared to sedentary control groups, while increasing hippocampal neurogenesis. Ibuprofen treatment reduces fatigue and depressive-like behaviors in tumor-bearing mice, while reducing IL-1β and IL-6 mRNA expression in the hippocampus, compared to healthy control mice (226). Furthermore, minocycline administration reduces central levels of pro-inflammatory cytokines and microglial activation, attenuating depressive-like behavior in tumor-bearing mice (55). Similarly, minocycline administration or functional blockade of a receptor expressed on myeloid cells attenuates cisplatin-induced CIPN (227) by suppressing the microglial pro-inflammatory response.

Alternative interventions include plant-derived adjuvant therapy drugs, such as those used in traditional Ayurvedic medicine (228). For example, pretreatment with rutin, astaxantin, or catechin significantly prevents the behavioral and neurobiological impairments induced by doxorubicin treatment in rodents (229–231). These bioceuticals also decrease TNF-α, prostaglandin E2, and COX-2 levels in hippocampus (230, 231). Furthermore, tetrahydrocurcumin exerts neuroprotective effects for vincristine-induced CIPN by decreasing oxidative stress, calcium and TNF-α levels in rats (232). These studies demonstrate the immunomodulatory, anti-inflammatory, and neuroprotective properties of these plant-based drugs in the context of chemotherapy.

CONCLUSION

The current review organizes and evaluates the evidence supporting how cancer and cancer treatments can influence neuroimmune pathways, leading to behavioral and neurobiological changes. Notable progress has been made in cancer diagnoses and treatment, prioritizing the need for understanding and intervention that addresses the mental welfare of cancer survivors. Additional basic science research using various modeling approaches is required to untangle and to understand the interactions among various cancer treatments and their paradigms, tumor biology, and stress. These models will be essential to determining the role of neuroimmune pathways in neuronal and behavioral consequences of cancer. Complementary neuroimmune-focused information is warranted in clinical research, potentially *via* postmortem brain autopsies, magnetic resonance spectroscopy, and further studies of anti-inflammatory interventions. Finally, the extent to which cancer-induced behavioral changes differ from the same changes in other disease contexts can contribute to the understanding of factors that influence onset versus persistence of these comorbidities in cancer patients and survivors. In summary, increasing recent basic and clinical science evidence points to potentially additive neuroimmune mechanisms due to various components of the cancer experience in cancer-associated behavioral comorbidities (depression, anxiety, fatigue, cognitive disturbances, and neuropathic pain).

AUTHOR CONTRIBUTIONS

The authors contributed equally for conceptualization, data synthesis, and manuscript preparation of this review.

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Distinct Gene Profiles of Bone Marrow-Derived Macrophages and Microglia During Neurotropic Coronavirus-Induced Demyelination

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Multiple Sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS) characterized by demyelination and axonal loss. Demyelinating lesions are associated with infiltrating T lymphocytes, bone marrow-derived macrophages (BMDM), and activated resident microglia. Tissue damage is thought to be mediated by T cell produced cytokines and chemokines, which activate microglia and/or BMDM to both strip myelin and produce toxic factors, ultimately damaging axons and promoting disability. However, the relative contributions of BMDM and microglia to demyelinating pathology are unclear, as their identification in MS tissue is difficult due to similar morphology and indistinguishable surface markers when activated. The CD4 T cell-induced autoimmune murine model of MS, experimental autoimmune encephalitis (EAE), in which BMDM are essential for demyelination, has revealed pathogenic and repair-promoting phenotypes associated with BMDM and microglia, respectively. Using a murine model of demyelination induced by a gliatropic coronavirus, in which BMDM are redundant for demyelination, we herein characterize gene expression profiles of BMDM versus microglia associated with demyelination. While gene expression in CNS infiltrating BMDM was upregulated early following infection and subsequently sustained, microglia expressed a more dynamic gene profile with extensive mRNA upregulation coinciding with peak demyelination after viral control. This delayed microglia response comprised a highly pro-inflammatory and phagocytic profile. Furthermore, while BMDM exhibited a mixed phenotype of M1 and M2 markers, microglia repressed the vast majority of M2-markers. Overall, these data support a pro-inflammatory and pathogenic role of microglia temporally remote from viral control, whereas BMDM retained their gene expression profile independent of the changing environment. As demyelination is caused by multifactorial insults, our results highlight the plasticity of microglia in responding to distinct inflammatory settings, which may be relevant for MS pathogenesis.

Keywords: macrophages, microglia, central nervous system, demyelination, viral encephalomyelitis

Abbreviations: BMDM, bone marrow-derived macrophages; CNS, central nervous system; EAE, experimental autoimmune encephalitis; MHC, major histocompatibility complex; mAb, monoclonal antibody; MHV, mouse hepatitis virus; MS, multiple sclerosis; NO, nitric oxide; ROS, reactive oxygen species; OLG, oligodendrocytes; TMEV, Theiler's murine encephalomyelitis virus; TNF, tumor necrosis factor; WT, wild-type.

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INTRODUCTION

Multiple Sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS), characterized by demyelination and axonal damage. Active demyelinating lesions are characterized by CD8 T cells, CD4 T cells expressing both Th1 and Th17 cytokines, bone marrow-derived macrophages (BMDM) and activated CNS resident microglia (1, 2). Myeloid cells activated by T cell effector functions are thought to participate in tissue damage by removing or "stripping" myelin (3), and secreting toxic factors, such as reactive oxygen species, nitric oxide and the pro-inflammatory cytokines, tumor necrosis factor (TNF), and IL-1β (4, 5). Activated microglia also secrete chemokines, which recruit innate and adaptive immune cells into the parenchyma, further amplifying the destructive inflammatory response (5). However, both BMDM and microglia effector functions are highly heterogeneous depending on the environment and may not only contribute to disease progression but also to resolution (6, 7). For example, by removing apoptotic cells and debris, their phagocytic activity favors tissue repair and is essential for disease resolution (3). In addition, both cell populations secrete anti-inflammatory cytokines, such as IL-10 and TGF-β, as well as trophic factors, which provide an environment that promotes tissue repair and neuronal protection (8). The heterogeneity of the inflammatory response associated with MS lesions at the cellular and functional levels, thus makes it difficult to establish detrimental versus disease resolving functions of BMDM and microglia in MS pathogenesis. In addition to the inherent limitations associated with sampling CNS tissues for longitudinal studies, the individual role of BMDM versus microglia as pathological mediators remains ambiguous due to morphological similarities and lack of reagents uniquely identifying each population. However, increasing evidence from animal models supports the concept that microglia and BMDM comprise two effector populations with distinct origins (derived from progenitors in the embryonic yolk sac and circulating monocytes respectively) and functions during MS and other neuroinflammatory disorders (9).

A variety of murine models, including autoimmune- and viral-induced demyelination, have been developed to study pathogenic features of MS (10). The most common is the experimental autoimmune encephalomyelitis (EAE), an autoreactive CD4 T cell-induced autoimmune demyelination characterized by infiltration of myelin-specific Th1 and Th17 cells, BMDM and microglial activation (11, 12). Pathogenesis during EAE is associated with temporally distinct microglial activation and BMDM CNS infiltration. Early microglia activation is insufficient to trigger clinical disease, whereas delayed CNS recruitment of BMDM directly correlates with disease progression. Importantly, depletion of BMDM but not microglia inhibits EAE (13, 14). Similarly, mice deficient in CCL2 ($CCL2^{-/-}$), a chemokine essential for inflammatory monocyte recruitment into the CNS (15), are resistant to EAE (16). In support of detrimental BMDM functions, a combined histological and gene profiling study showed that demyelination is mediated by BMDM associated with nodes of Ranvier, whereas debris clearance is achieved by microglia (17). Altogether, studies in the EAE model demonstrate that BMDM recruitment into the CNS is essential for the process of myelin loss and clinical manifestation.

Inflammatory demyelination is also induced following infection with two natural viral mouse pathogens, Theiler's murine encephalomyelitis virus (TMEV) and members of the neurotropic mouse hepatitis viruses (MHV). TMEV infection induces an autoimmune disease in which BMDM are essential for both viral persistence and demyelination (18, 19). However, the function of BMDM as a main reservoir of active viral replication during chronic TMEV infection, limits efforts to assess their role in demyelination independent of virus load (20). In contrast, infection with the non-lethal glia tropic MHV strain designated JHMV predominantly targets oligodendrocytes (OLG) and to a lesser extent microglia and astrocytes. Viral replication peaks at day 5 post infection (p.i.), but infectious virus is reduced below detection by day 14 p.i. Acute infection initiates rapid CNS recruitment of predominantly BMDM, but also neutrophils and NK cells, followed by infiltration of both CD8 and CD4 T cells, as observed in active MS lesions. The T cell response, which is essential to reduce viral replication, is highly Th1 polarized with no evidence of IL-17 or GM-CSF production (21–23). Importantly, T cell-mediated virus control coincides with initiation of demyelination, which peaks between days 14–21 p.i. after infectious virus is cleared (24, 25). Although OLG tropism is a requirement for demyelination, immunodeficient mice demonstrated that infection of OLG in the absence of adaptive immunity is insufficient to cause demyelination. However, transfer of either virus-specific CD4 or CD8 T cells into virus infected immunodeficient mice leads to demyelination (26, 27). Furthermore, IFN-γ dependent control of infectious virus within OLG and no evidence for OLG apoptosis, suggested that direct T cell-mediated cytolysis of OLG does not play a major role in myelin loss (28). This implicates T cell activated BMDM and microglia as the most probable mediators of myelin destruction. Moreover, both myeloid populations are abundant in lesions and occasionally associated with damaged axons (29). However, in contrast to EAE, genetic or chemical depletion of monocytes during JHMV infection does not alter disease severity, virus replication or myelin loss (30, 31), suggesting that BMDM are dispensable for JHMV-induced demyelination.

This study takes advantage of the distinct tissue environments established during EAE and JHMV infection to characterize temporal alterations in gene expression profiles of BMDM versus microglia in a Th1 biased demyelination model. To date, we are not aware of any reports evaluating the signature profile of microglia associated with pathogenic functions during demyelination. The results reveal that CNS infiltrating BMDM rapidly establish a characteristic profile including M1 and M2 markers, which prevails throughout infection as the population declines. By contrast, gene expression in microglia is only prominently altered remote from viral control concomitant with demyelination; distinct from BMDM, the gene expression pattern is skewed to a highly pro-inflammatory and phagocytic profile. The results overall highlight the plasticity of microglia responses in distinct inflammatory settings, which may be relevant for MS pathogenesis at distinct stages of disease.

MATERIALS AND METHODS

Mice

Wild-type (WT) C57BL/6 mice were purchased from the National Cancer Institute (Frederick, MD, USA). Homozygous CCL2 deficient ($CCL2^{-/-}$) mice were originally obtained from B. J. Rollins (Dana-Farber Cancer Institute, Boston, MA, USA). $CX3CR1^{GFP/GFP}$ (B6.129P-*Cx3cr1*^{tm1Litt}/J) and $CCR2^{RFP/RFP}$ (B6.129 (Cg)-*Ccr2*$^{tm2.1Ifc}$/J) mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and crossed to generate $CX3CR1^{GFP/+}CCR2^{RFP/+}$ mice. Transgenic mice were bred and maintained at the Biological Research Institute under sterile conditions. All procedures were preformed in compliance with the Cleveland Clinic Institutional Animal Care and Use Committee approved protocols.

Virus and Infections

The glia tropic JHMV neutralizing monoclonal antibody (mAb)-derived 2.2v-1 variant was used for all infections (32). Mice of both sexes between 6 and 7 weeks of age were infected in the left hemisphere with 1,000 PFU of JHMV diluted in endotoxin-free Dulbecco's phosphate-buffered saline (PBS) in a final volume of 30 µl. Mice were monitored daily for clinical disease severity according to the following scale: 0, healthy; 1, hunched back and ruffled fur; 2, partial hind limb paralysis or inability to maintain the upright position; 3, complete hind limb paralysis; 4, moribund or dead.

Isolation of CNS Mononuclear Cells, Flow Cytometry, and Cell Sorting

For analytical flow cytometry, anesthetized mice were perfused with ice-cold PBS, and resected brains and spinal cords homogenized using a Ten-Broeck tissue grinder as described (33). Tissue homogenates were adjusted to 30% percoll (Pharmacia, Uppsala, Sweden) and underlaid with 1 ml 70% percoll prior to centrifugation at 850 *g* for 30 min at 4°C. CNS mononuclear cell were recovered from the 30/70% interface, washed and resuspended in FACS buffer (PBS + 1% bovine serum albumin). Cells were blocked with anti-mouse CD16/CD32 (clone 2.4G2) mAb for 15 min on ice prior to staining. Staining was performed for 30 min on ice using fluorescein isothiocyanate, phycoerythrin, peridin chlorophyll protein complex (PerCP), or allophycocyanin (APC) conjugated mAb (all from BD Biosciences except where indicated) specific for CD45 (clone Ly-5), CD11b (clone M1/70), F4/80 (Serotec, Raleigh, NC, USA) and major histocompatibility complex (MHC) class II (clone 2G9). Cells were then washed twice in FACS buffer prior to analysis using a BD Accuri flow cytometer (BD Biosciences) and FlowJo software (Tree Star Inc., Ashland, OR, USA).

For cell purification, spinal cords from PBS-perfused $CX3CR1^{GFP/+}CCR2^{RFP/+}$ mice were finely minced with a razor blade. Minced tissues were enzymatically digested in RPMI 1640 medium containing 10% fetal calf serum, 0.5% collagenase D (100 mg/ml) Roche, Basel, Switzerland and 1% DNase I (1 mg/ml) (Sigma Aldrich, St. Louis, MO, USA) for 40 min at 37°C. Collagenase was then inactivated by addition of 1% 0.1 M EDTA for 5 min at 37°C prior centrifugation at 400 *g* for 7 min at 4°C. Spinal cord-derived cells from seven mice were pooled and isolated using percoll gradients as described above and then stained with CD11b-PerCP and CD45-APC for 30 min on ice. Spinal cord-derived BMDM ($CD45^{hi}CD11b^{+}CCR2^{RFP+}$) and microglia ($CD45^{low}CD11b^{+}CX3CR1^{GFP+}$) were purified using a FACSAria cell sorter (BD Biosciences) and resuspended in Trizol. Yields from 7-pooled mice ranged between 5.4–20 × 10^5 cells for BMDM and 0.5–1.2 × 10^5 cells for microglia depending on the time p.i. Microglia from naïve mice were used to assess baseline expression, whereas circulating monocytes were used as controls for CNS infiltrated BMDM after infection. Monocytes were isolated from blood treated with Gey's solution to lyse red blood cells prior to staining and cell sorting.

Gene Expression Profiling Using nCounter Analysis

RNA was prepared by extraction with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and Direct-zol RNA mini prep (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions. Gene expression profiles were analyzed using the nCounter mouse Myeloid Innate Immune panel comprising 754 targets representing all major myeloid cell types and generated according to the manufacturer's protocol (NanoString Technologies, Seattle, WA, USA). The NanoString nCounter system directly captures and counts individual mRNA transcripts using a multiplexed measurement system thereby omitting cDNA based amplification (34). Analysis was performed using nSolver Analysis Software v3.0 and Ingenuity pathway analysis (Qiagen, Hilden, Germany). Venn diagrams from individual gene lists and protein-protein interaction networks were constructed using Genespring (Agilent, Inc.) and STRING software (http://www.string-db.org).

Reverse Transcription and Real-Time PCR

To confirm validity of Nanostring nCounter analysis, a small set of selected genes were analyzed by real-time PCR (Figure S1 in Supplementary Material). Following RNA extraction as described above, first-strand cDNA was synthesized using reverse transcriptase (Invitrogen) with oligo-dT and random primers (Promega, Madison, WI, USA) as described (35). Gene expression analysis was performed using a 7500 Fast real-time PCR system (Applied Biosystems, Foster city, CA, USA), SYBR Green Master mix (Applied Biosystems) and the following primers: GAPDH, 5′-CATGGCCTTCCGTGTTCCTA-3′ (forward) and 5′-ATGCCTGCTTCACCACCTTCT-3′ (reverse); IL15, 5′-TGAGGCTGGCATTCATGTCTT-3′ (forward) and 5′-TCCAGTTGGCCTCTGTTTTAGG-3′ (reverse); IL1rn 5′-AGATAGACATGGTGCCTATTGACCTT-3′ (forward) and 5′-CATCTCCAGACTTGGCACAAGA-3′ (reverse) and Arg1 5′-TGGGTGGATGCTCACACTGA-3′ (forward) and 5′-CAGGTTGCCCATGCAGATT-3′ (reverse). Transcripts levels were normalized to the housekeeping gene GAPDH and converted to a linearized value using the following formula: $2^{(C_T GAPDH - C_T gene)} \times 1{,}000$, where C_T represents the threshold cycle value.

Histological Analysis

Following PBS perfusion, spinal cords were fixed in 10% neutral buffered Formalin, embedded in paraffin and sections stained with Luxol Fast Blue as described to visualize demyelination (36). For analysis of Iba1$^+$ cells spinal cords from ice-cold PBS-perfused mice were quickly embedded in OCT and kept at −80°C until 10 µm sections were prepared. Sections were fixed with paraformaldehyde for 20 min, treated with blocking solution for 30 min and then stained with rabbit anti-Iba1 mAb (Wako, Osaka, Japan) overnight at 4°C. Goat anti-rabbit secondary Ab (Invitrogen) was added for 1 h at room temperature and sections mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA). Sections were analyzed using a Leica TCS confocal microscope.

RESULTS

Infiltrating Macrophages Are Dispensable to JHMV-Induced Demyelination

To better characterize reactivity of microglia and infiltrating BMDM following JHMV infection, we initially monitored CNS infiltration of BMDM, as well as upregulation of MHC class II as an activation marker on both CNS BMDM and microglia by flow cytometry. BMDM with a typical CD45^{hi}CD11b$^+$F4/80$^+$ phenotype comprised the majority of inflammatory leukocytes as early as day 3 p.i. and then progressively decreased as virus replication is controlled by T cells. At the onset of demyelination at day 10 p.i., the BMDM population stabilized at ~10% of the infiltrating leukocytes (**Figure 1A**). BMDM initially infiltrated as MHC class II^{lo} expressing cells, but the vast majority upregulated MHC class II by day 7 p.i. MHC class II expression on microglia was sparse at days 3 and 5 p.i., but rapidly increased by day 7 p.i. and then gradually declined by day 14 p.i. (**Figure 1A**). These kinetics supported that microglia and BMDM activation peaks delayed relative to peak BMDM accumulation and coincides with peak T cell IFN-γ production (36, 37). Enhanced activation of microglia at day 7 p.i., compared to earlier times p.i., was also supported by progression of morphological changes, evidenced by enlarged cell bodies and retracted and thickened processes (**Figure 1B**). The decline of BMDM, but an ongoing activation phenotype of microglia at the time of evident demyelination implicated microglia as mediators of tissue damage during JHMV encephalomyelitis.

Biochemical depletion of peripheral monocytes indeed supported that BMDM are not essential to tissue destruction in JHMV-infected mice (30). Data from our own laboratory further demonstrated that the chemokine CCL2 is essential for BMDM accumulation within the CNS (31). The absence of CCL2 resulted in an ~80% reduction of BMDM at all time points, including day 14 p.i. (31) and (**Figure 1C**) when demyelination is prominently evident in WT mice. Nevertheless, microglia activation, as monitored by MHC class II expression, was independent of CCL2 (**Figure 1D**). Most importantly, the absence of CCL2-dependent BMDM within the CNS did not alter demyelination (**Figure 1E**). Similar myelin loss at day 21 p.i. comparing WT and CCL2$^{-/-}$ infected mice supported the concept that microglia mediate demyelination during JHMV infection.

Characterization of BMDM and Microglia Under Homeostatic Conditions

We next evaluated effector functions of BMDM versus microglia associated with JHMV-induced demyelination by comparing gene expression profiles using nCounter analysis of mRNA isolated from purified BMDM and microglia of infected CX3CR1$^{GFP/+}$CCR2$^{RFP/+}$ mice. Characteristic expression of CX3CR1^{GFP} and CCR2^{RFP} on CD45^{high}CD11b$^+$ BMDM (population #1) and CD45^{low}CD11b$^+$ microglia (population #2) is shown in **Figure 2** throughout days 5–14 p.i. Microglia were characterized by high expression of CX3CR1 and undetectable CCR2 expression (**Figures 2B,C**) similar to other inflammatory models (17, 38). In contrast, CNS infiltrating BMDM expressed CCR2 and low levels of CX3CR1 compared to microglia (**Figure 2B**). Co-expression of CCR2 and CX3CR1 was maintained on BMDM at all time points p.i. and no CX3CR1$^-$ cells were detectable (**Figure 2C**).

As both microglia and infiltrating BMDM retained their phenotype throughout infection, CD45^{low}CD11b$^+$CX3CR1^{GFPhi}CCR2$^-$ and CD45^{hi}CD11b$^+$CX3CR1^{GFPlow}CCR2$^+$ populations were isolated by FACS from spinal cords at days 5, 7, 10, and 14 p.i. for subsequent mRNA expression analysis. Age-matched naïve animals were used to isolate microglia and blood circulating monocytes as precursors of CNS-infiltrating BMDM. Gene expression profiles for all purified populations were obtained using nCounter analysis and the Innate Myeloid Immune panel. The respective naïve populations were used to assess signature gene expression profiles under homeostatic conditions (**Figure 3**).

Figure 3A shows the top 50 highly expressed genes within each population relative to three nCounter platform housekeeping genes, namely G6pdx, Polr1b, and Tbp, selected for three high, medium, and low expression, respectively, in this part of analysis platform. **Figure 3B** lists the top 50 enriched genes specific for microglia compared to monocytes, or monocytes versus microglia, respectively. Among the top 50 genes highly expressed in microglia, 15 were also specific and included genes of the complement cascade (C1qa, C1qb, C1qc, and C3ar1) and Trem2, encoding a cell surface receptor involved in phagocytic functions and known to be expressed by microglia (39). Other genes, such as Adamts1, F11r or Hpgds, found within the top 50 enriched genes expressed by microglia (**Figure 3B**) were also previously described as microglia specific (17, 40). Cx3cr1 mRNA encoding the fractalkine receptor and used as a marker for microglia (41), was also among the top 50 highly expressed genes (**Figure 3A**), but not unique, consistent with the CX3CR1^{lo} phenotype on circulating monocytes. Similarly, CCR2 expression characteristic of monocytes was confirmed by ccr2 mRNA as the second in place of the top 50 expressed genes specific for circulating monocytes (**Figure 3B**). Other specific signature genes of monocytes are related to motility and migration/tissue invasion, e.g., S100a4, S100a8, S100a9, Fn1, Sema4, Mmp8, and to a lesser extent MHC class II antigen presentation, e.g., H2-Ab1, CD74, and Fas. Microglia and circulating monocytes also shared 17 highly expressed genes, including genes characteristic of the myeloid lineage such as Csf1r, a gene coding for a cell surface receptor essential for hematopoietic

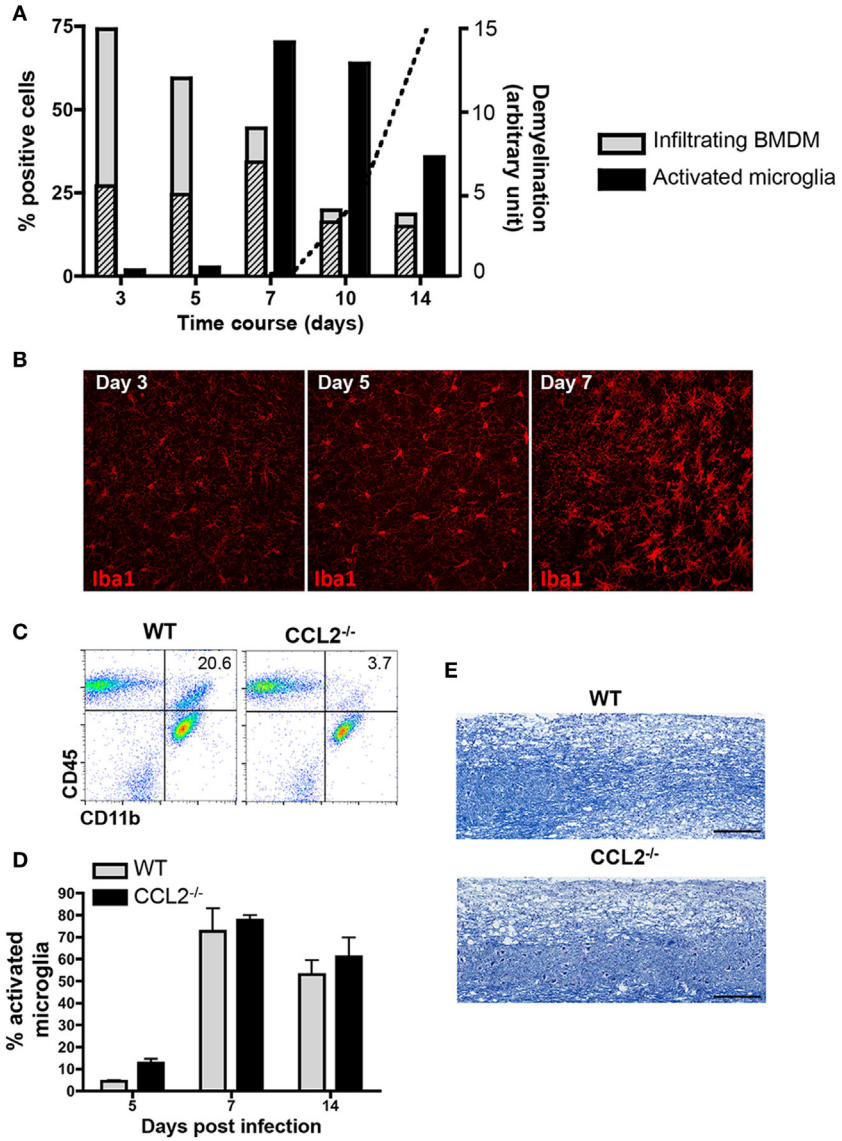

FIGURE 1 | Demyelination correlates with microglia activation and is not affected in absence of bone marrow-derived macrophage (BMDM) central nervous system (CNS) infiltration. **(A)** Brains dissected from JHMV-infected wild-type (WT) mice were analyzed by flow cytometry for infiltrating CD11b^{+}F4/80^{+} BMDM and activated CD45^{low}CD11b^{+} microglia defined by their major histocompatibility complex (MHC) Class II^{+} phenotype between days 3 and 14 p.i. Gray bars depict the percentage of total CD11b^{+}F4/80^{+} BMDM within infiltrating CD45^{hi} leukocytes, with striped bars representing the MHC Class II expressing BMDM fraction. Black bars represent the proportion of Class II^{+} cells within the microglia population. Data are from three-pooled mice per time point and representative of at least three experiments. The superimposed dotted line shows the relative kinetics and extent of demyelination in arbitrary units. **(B)** Microglia morphological changes at days 3, 5, and 7 p.i. were visualized by confocal microscopy of brain sections stained with anti-Iba1 monoclonal antibody (mAb). 40× magnification. Pictures are representative of four separate animals. **(C)** Brains of WT and CCL2$^{-/-}$ infected mice at day 14 p.i. were analyzed for CD45 and CD11b expressing myeloid cells. Representative flow cytometry plots show a reduction of CD11b^{+} cells within the infiltrating CD45^{hi} population in the absence of CCL2. Numbers represent the percentage of CD11b^{+} cells within CD45^{hi} infiltrates. **(D)** Brains of infected WT and CCL2$^{-/-}$ mice between days 5 and 14 p.i. were analyzed by flow cytometry for activated MHC Class II^{+} microglia Bar graphs depict the percentage of class II + cells within CD45^{low}CD11b^{+} cells. Data represent the mean ± SEM of three experiments with n = 3-pooled mice per group per experiment. **(E)** Demyelinating lesions within spinal cords of WT and CCL2$^{-/-}$ mice at day 21 p.i. were visualized by Luxol Fast Blue staining.

precursors differentiation into myeloid cells and Mpeg1, a gene coding for a membrane protein with a perforin domain expressed on myeloid cells (**Figure 3A**). Altogether, these results highlight unique as well as common basal expression signatures of each purified myeloid population, thus providing a basis for characterization of altered expression patterns following JHMV infection.

BMDM and Microglia Display Overall Distinct Patterns of Gene Expression as Well as Temporal Regulation Throughout JHMV Infection

Following JHMV infection, the majority of commonly expressed genes in microglia and circulating monocytes were regulated

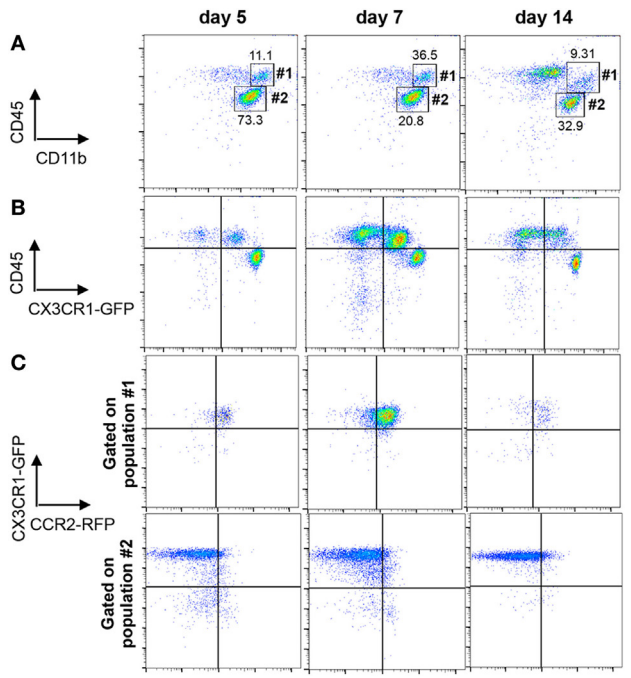

FIGURE 2 | Distinction of microglia and infiltrating bone marrow-derived macrophage (BMDM) within the central nervous system (CNS) of CX3CR1$^{GFP/+}$CCR2$^{RFP/+}$ mice. **(A)** Representative flow cytometry plots of CD45^{hi}CD11b^{+} BMDM (population #1) and CD45^{low}CD11b^{+} microglia (population #2) gated on total CD45 cells from JHMV-infected spinal cords at the indicated time points. **(B)** Cells from Panel A were analyzed for differential CX3CR1^{GFP} expression on CD45^{hi} and CD45^{low} populations. **(C)** Cells from Panel A gated on CD45^{hi}CD11b^{+} BMDM (population 1) or CD45^{low}CD11b^{+} microglia (population #2) were assessed for CX3CR1^{GFP} and CCR2^{RFP} expression. All data are acquired from mechanically disrupted tissue of JHMV-infected CX3CR1$^{GFP/+}$CCR2$^{RFP/+}$ mice and representative of two separate experiments with at least three mice per time point per experiment.

similarly within the CNS over time. For example, Selpig mRNA was downregulated at all time points during JHMV infection, while Apoe was specifically upregulated at days 10 and 14 p.i. in both BMDM and microglia (**Figure 4A**). Interestingly however, three genes among the common and highly expressed genes were regulated differently. Ctss mRNA, encoding for Cathepsin S, a lysosomal cysteine proteinase participating in the MHC Class II molecule antigen presentation pathway as well as nociception (42, 43), was specifically upregulated within BMDM, with highest levels observed at days 10 and 14 p.i. (**Figure 4A**), when demyelination increases. By contrast, microglia transiently downregulated Ctss mRNA at day 7 p.i. Opposite regulation was also observed for Dusp1 mRNA (**Figure 4A**). Dusp1 mRNA encodes the dual specificity protein phosphatase 1, an enzyme involved in the cellular stress response and a negative regulator of cell proliferation (44). While Dusp1 mRNA levels were vastly upregulated early following BMDM accumulation, but declined by day 7 p.i. and thereafter, levels were rapidly downregulated within microglia (**Figure 4B**). Finally, Tyrobp mRNA encoding for the TREM2 adaptor DAP12 and known to regulate microglia phagocytic functions (39), was downregulated in BMDM throughout infection, but specifically upregulated within microglia at days 10 and 14 p.i. (**Figure 4B**). This expression pattern on microglia correlated with the onset of myelin loss and supported TREM2 signaling specifically by microglia in response to tissue damage.

To determine whether apparently differential functions of BMDM and microglia associated with JHMV-induced demyelination are reflected in distinct gene profiles, we monitored overall up- and downregulation of gene expression relative to basal levels in each population. Analysis times were chosen to correlate with innate responses (d5 p.i.), peak T cell effector function (d7 p.i.), resolution of infection and initiation of demyelination (d10 p.i.), and finally viral clearance and overt demyelination (d14 p.i.). At day 5 p.i. a higher number of genes were differentially regulated within infiltrating BMDM compared to microglia (231 versus 76; **Figure 5A**). Moreover, almost 80% of the genes showing altered expression in early infiltrated BMDM were increased compared to basal levels, while only 54% were increased in microglia; the remaining differentially expressed mRNAs were decreased (**Figure 5B**). The overall number of differentially expressed genes slightly declined in BMDM by day 7 p.i., when T cells exert maximal effector function (37), and remained fairly constant throughout day 14 p.i. (**Figure 5A**). Furthermore, the relative decline in the proportion of upregulated mRNAs coincided with an increased proportion of downregulated genes, reaching a roughly equal distribution at days 10–14 p.i., when virus is largely controlled (**Figure 5B**). In contrast, microglia altered their gene expression pattern extensively at day 7 p.i. (97 genes, **Figure 5A**) with 67% of differentially regulated genes showing increases (**Figure 5B**). By day 10 p.i., overall altered gene expression remained stable relative to day 7 p.i., with equal proportions showing increases and decreases. However, as myelin loss progresses by day 14 p.i., differentially regulated genes increased again in numbers, with the proportion of upregulated mRNAs reaching 95% (**Figure 5A**). Altogether these data show unique regulation of gene profiles in BMDM compared to microglia throughout the course of infection. While most changes were evident in BMDM following initial CNS accumulation, microglia revealed most pronounced changes at the time of myelin loss.

We further analyzed differential gene expression across time points focusing on upregulated genes using Venn diagrams to reveal the relative proportion of genes that were commonly increased at all time points (**Figure 5C**). Of the 183 upregulated genes in BMDM at day 5 p.i., 62 were unique to day 5. On the other hand, 77 genes (representing 42% of all upregulated genes) remained highly expressed at all other time points (**Figure 5C**). Of note, not a single gene transcript was specifically upregulated at day 7 p.i., and only three overlapped with sustained upregulation at days 10 and 14 p.i. Similarly, only 4 gene transcripts were specifically elevated at day 10 p.i., 7 were unique to both days 10 and 14, and only one was unique to day 14 p.i. These results indicate that the gene expression profile characterizing BMDM is established early following infection, with sparse unique alterations as BMDM decline during infection. In stark contrast, only 3 of 41 gene transcripts upregulated in microglia at day 5 p.i. were unique to day 5, and no gene transcript was commonly upregulated across all time points analyzed. Furthermore, distinct from BMDM, 59 gene transcripts were

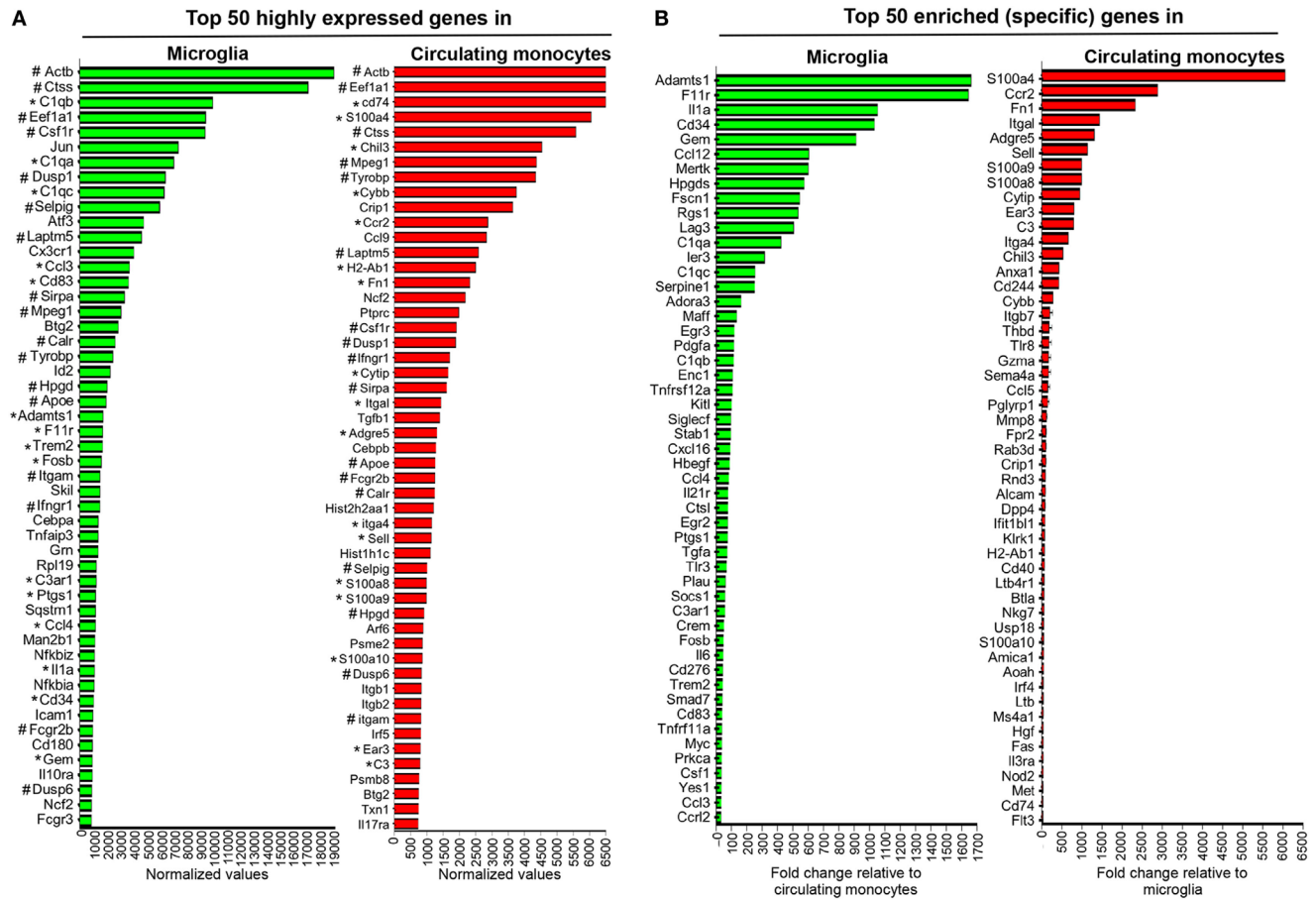

FIGURE 3 | Gene expression characterizing microglia and circulating monocytes under homeostatic conditions. Spinal cord-derived microglia ($CD45^{low}CD11b^{+}CX3CR1^{GFP+}$) and circulating blood monocytes ($CD11b^{+}CCR2^{RFP+}$) were purified from naïve $CX3CR1^{GFP/+}CCR2^{RFP/+}$ mice by FACS and RNA subjected to nCounter analysis using the myeloid cell probe panel. Panel **(A)** depicts the top 50 highly expressed genes and **(B)** the enriched genes uniquely characterizing each population. In **(A)** * highlights genes that are both highly expressed and enriched in each population, while # highlights genes highly expressed and common to both microglia and circulating monocytes.

uniquely upregulated by day 7 p.i., with none common to day 10 p.i., and only six overlapping with those upregulated at day 14 p.i. Although no gene transcripts were upregulated uniquely at day 10 p.i., 22 overlapped with those upregulated at day 14 p.i. A further 76 genes, comprising 53% of all upregulated genes at day 14 p.i., were specifically expressed at elevated levels at day 14 p.i. coinciding with overt myelin loss (**Figure 5C**). These profiles reveal a dynamic range of responses and extensive plasticity of gene expression profiles in microglia throughout JHMV infection (**Figure 5C**).

Gene Expression Profiles Characterizing BMDM and Microglia at Peak of Demyelination

We next used a protein–protein network connection constructed based on differential gene expression to specifically examine upregulation of gene transcripts within BMDM and microglia correlating with demyelination at day 14 p.i. For comparison, we also analyzed the network connection at day 5 p.i., when expression profiles were most prominently altered in BMDM, but more modestly in microglia. This comparative analysis was chosen to provide clues about specific functions and involvement of microglia relative to BMDM in tissue destruction (**Figures 6** and **7**). Our initial focus was on temporally altered networks in BMDM (**Figure 6**). At day 5 p.i., early infiltrated BMDM expressed a wide array of chemokines regulating CNS infiltration of both innate (CXCL2, CCL2, CCL4, CCL5, CCL7, and CCL12) and adaptive (CXCL9, CXCL10) immune cells (**Figure 6A**). A large cluster of molecules regulating the innate immune response, essential to limit early viral replication (45, 46), was also expressed by BMDM (**Figure 6A**). These include pathogen recognition receptors such as TLRs (TLR1-4 and TLR9) and molecules linked to the TNF pathway (TNF, TRAF6, etc.). Finally, BMDM expressed molecules involved in antigen presentation, including Tap1 and Tap2, as well as T cell activating co-stimulatory molecules (CD80 and CD86) or IL-12, which induce Th1 differentiation (**Figure 6A**). These results indicate that early infiltrating BMDM orchestrate the acute innate immune response crucial for limiting CNS viral spread, as well as initiating the adaptive immune response by recruiting

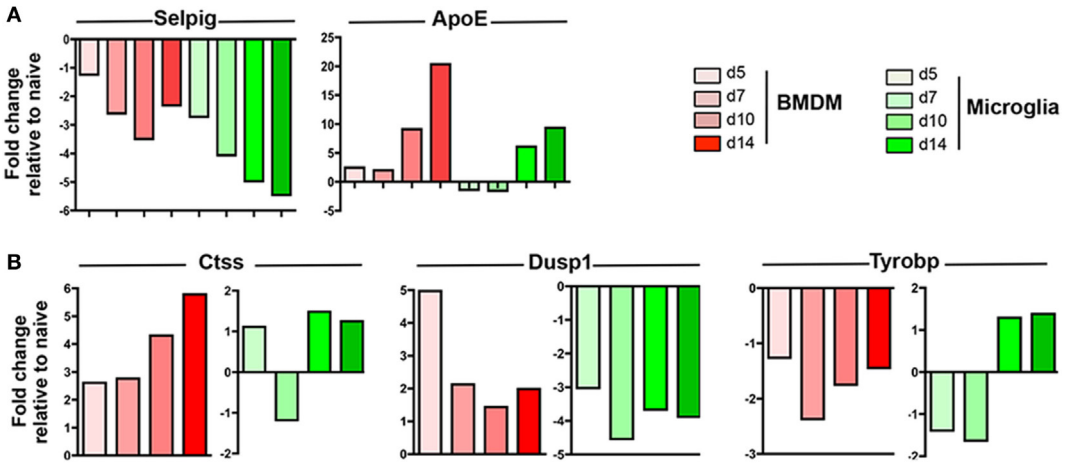

FIGURE 4 | Expression profiles of genes commonly expressed by central nervous system (CNS) infiltrating bone marrow-derived macrophage (BMDM) and microglia during JHMV infection. FACS purified BMDM and microglia from spinal cords of JHMV-infected JHMV-infected CX3CR1$^{GFP/+}$CCR2$^{RFP/+}$ mice were assessed for differential gene expression by nCounter analysis using the myeloid cell probe panel. **(A)** Selpig and ApoE genes were chosen as representative genes showing similar expression patterns in BMDM and microglia **(B)** Ctss, Dusp1, and Tyrobp genes were chosen to highlight distinct regulation in BMDM and microglia throughout infection. Data are obtained from the spinal cords of seven-pooled mice for each time point and represent the fold increase relative to naïve levels at days 5, 7, 10, and 14 p.i. in BMDM and microglia.

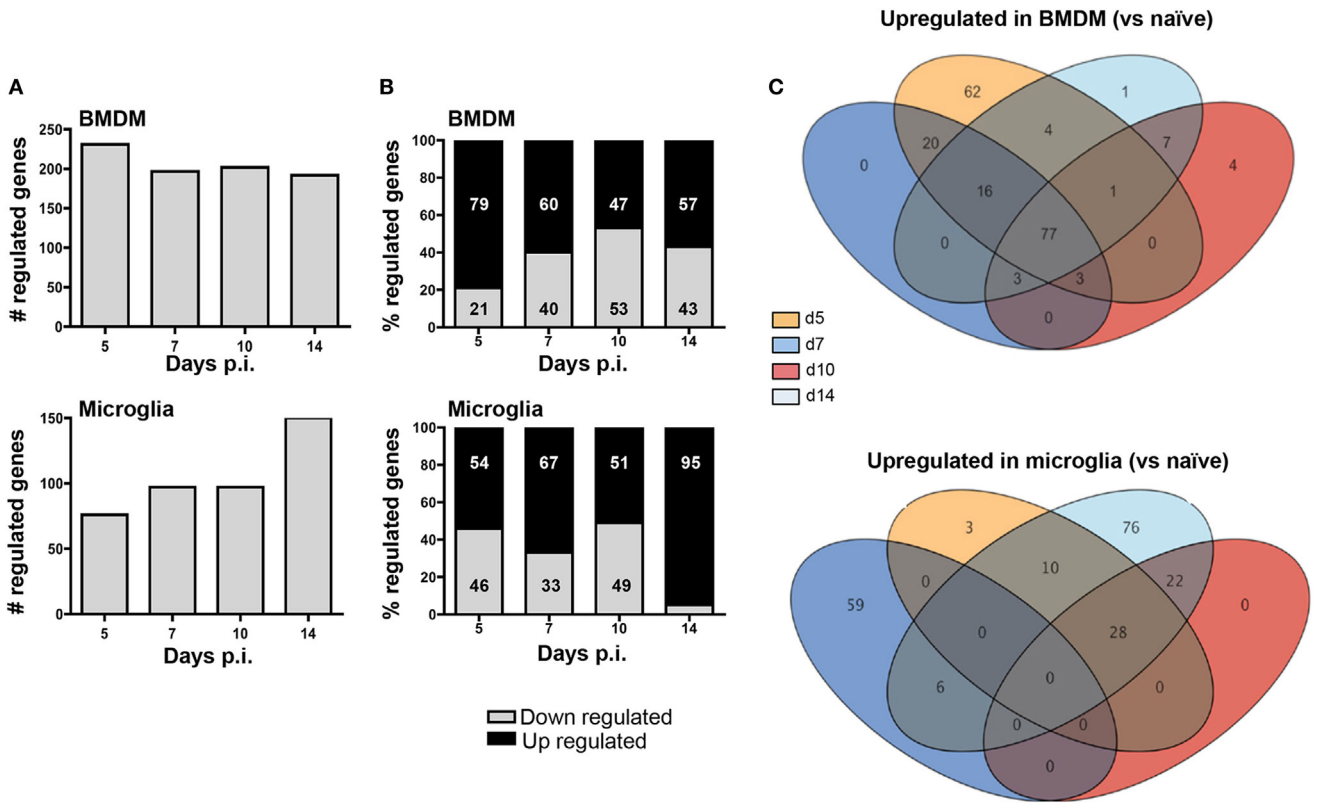

FIGURE 5 | Infiltrating bone marrow-derived macrophage (BMDM) and microglia reveal distinct gene regulation following JHMV infection. BMDM and microglia gene expression patterns obtained from infected mice described in **Figure 4** were analyzed for **(A)** the number of total regulated genes and **(B)** the relative distribution of up- and downregulated genes (percentage of increased and decreased) within each population. **(C)** The Venn diagram represents the number of genes upregulated at least twofold relative to the naïve populations throughout days 5, 7, 10, and 14 p.i. in BMDM and microglia. Numbers in overlapping ellipses represent genes upregulated at several time points.

and activating T cells. At day 14 p.i., correlating with peak demyelination, a more restrained number of mRNA transcripts were upregulated in BMDM (**Figure 6B**). The cluster of chemokines mobilizing immune cells was sustained (**Figure 6B**). In contrast, the molecular network extended from TNF was more limited at d14 p.i. compared to d5 p.i. (**Figures 6A,B**). Molecules regulating primarily the CD4 T cell response were expressed at day 14 p.i. and comprised gene transcripts involved in antigen presentation including MHC class II (H2-Ab1) and co-stimulatory molecules such as CD80 and CD86 (**Figure 6B**). Interestingly, among the more restricted number of gene transcripts upregulated at day 14 p.i. in BMDM, several were transcripts encoding M2 molecules, which included Arg1, Il1rn and Tgm2 (**Figure 6B**).

In contrast to the vast number of genes upregulated early following infection in BMDM, a significantly lower number of upregulated genes characterized microglia at day 5 p.i. (**Figure 7A**). Transcripts for chemokines regulating migration of both innate and adaptive immune cells, such as CCL2,

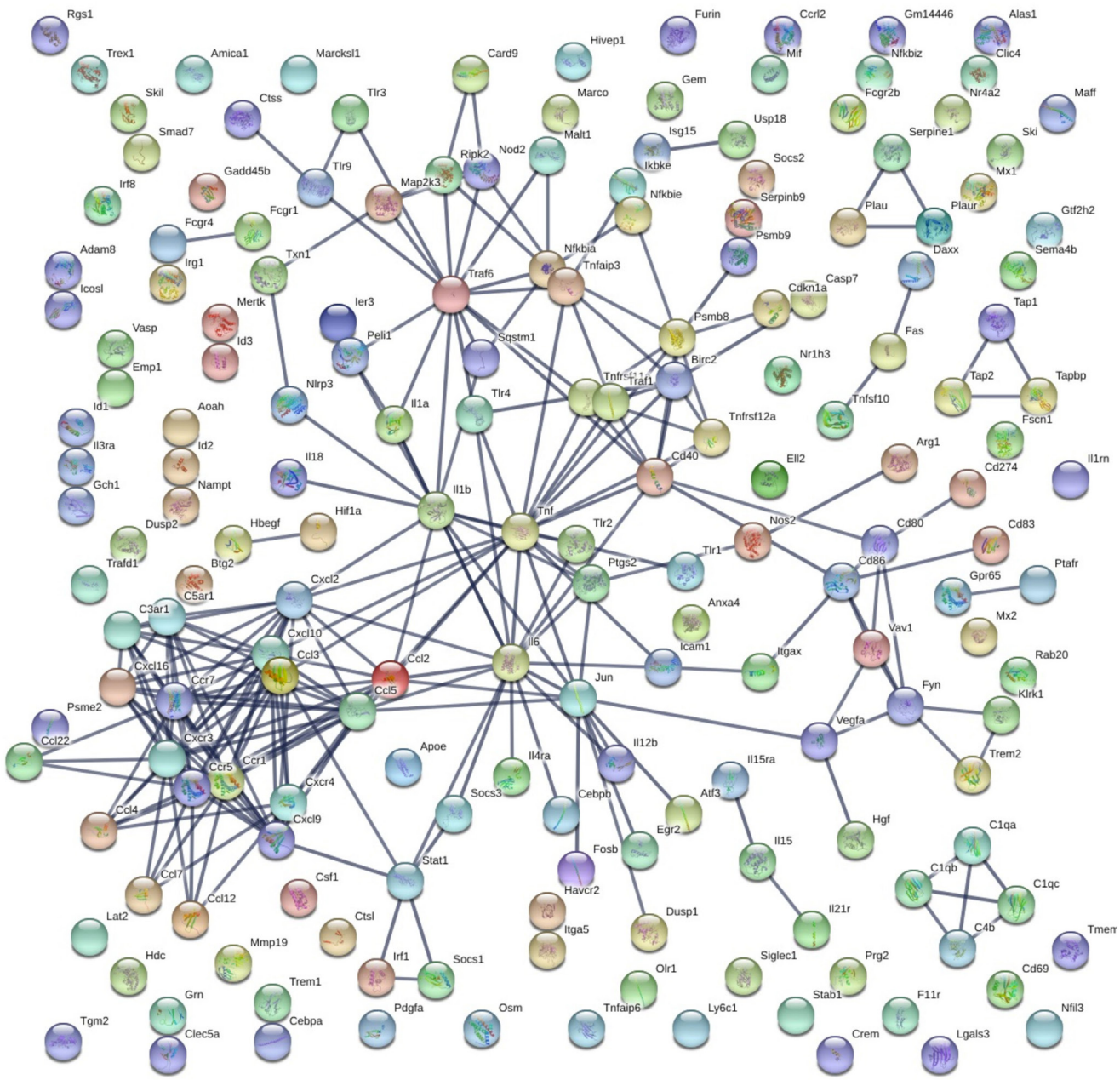

FIGURE 6 | Continued

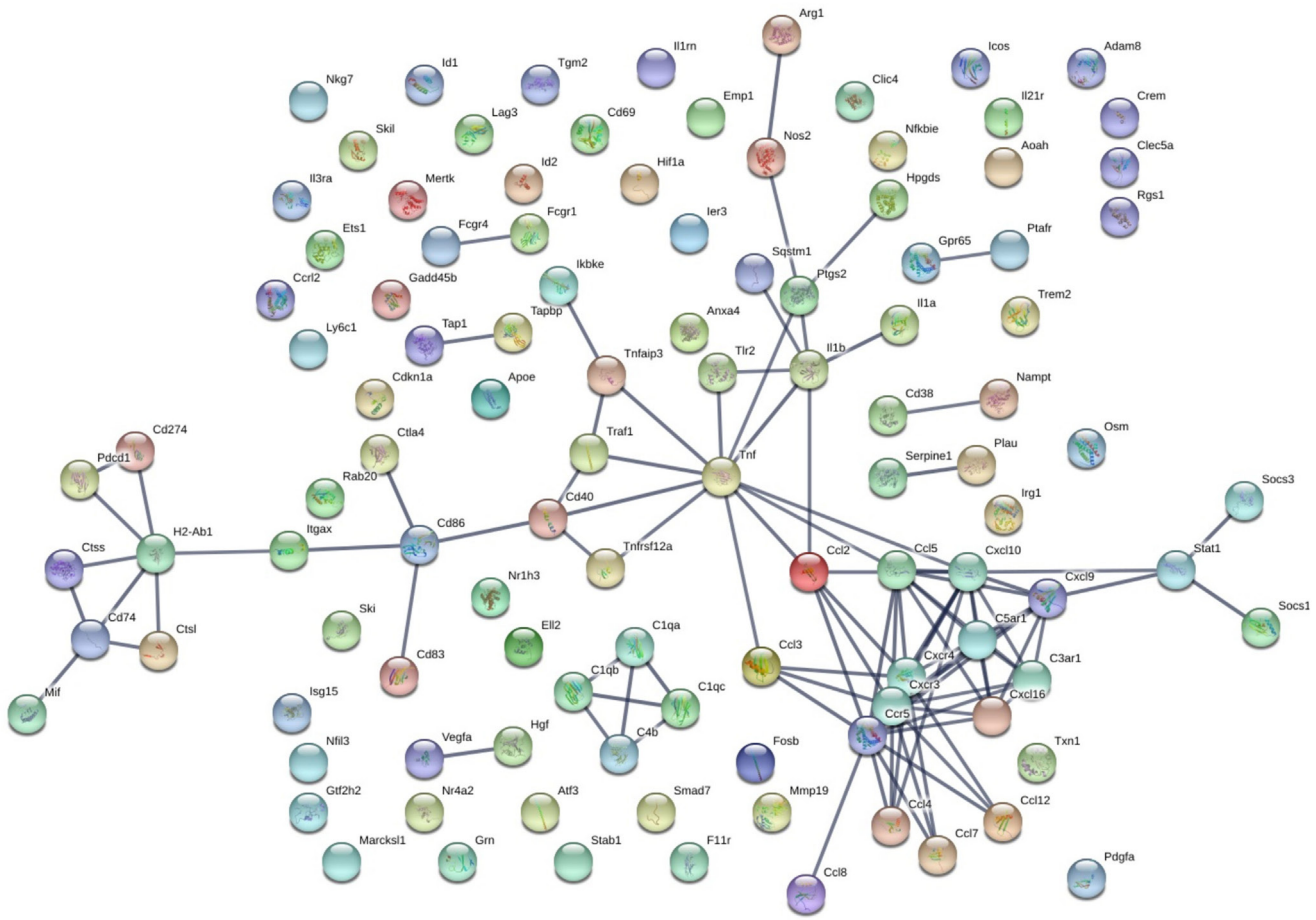

FIGURE 6 | Upregulated gene network within central nervous system (CNS) infiltrating macrophages at d5 and d14 p.i. Protein–protein network constructed based on genes upregulated by at least twofold in infiltrated bone marrow-derived macrophage (BMDM) at days 5 **(A)** and 14 **(B)** p.i. compared to naïve levels were analyzed using Ingenuity IPA software.

CCL3, CCL4, CCL12, CXCL10, and CXCL16, were expressed by microglia at day 5 p.i. (**Figure 7A**). Transcripts for TNF and other inflammatory cytokines generally associated with the innate responses, e.g., IL1a, IL1b, and IL18 were also upregulated early in microglia (**Figure 7A**). Another extended network of TNF comprised Psmb8 and Psmb9, subunits of the immunoproteasome, essential for antigen presentation by MHC class I molecules. By day 14 p.i., the number of upregulated transcripts extensively increased in microglia (**Figure 7B**). The most clustered network comprised proteins like CCL5, CXCL9, CXCL10, CXCL13, CXCL16, and CXCR4, all chemokines and chemokine receptors regulating migration and arrest of adaptive immune cells within the CNS during inflammation (47). This chemokine cluster was linked to TNF and inflammatory cytokines previously detected at d5 p.i., e.g., IL1a, IL1b, and IL18. Another extended network of TNF comprised Psmb8 and Psm9, also present at d5 p.i., Ctnnb1 encoding b-catenin, a cellular adhesion molecule, and cdkn1a, a cyclin inhibitor. Other upregulated gene transcripts associated with class I antigen presentation, e.g., tap1 and tap2, were also linked through a network associated with complement component genes (C3, C3ar1, C4b, C1qa, C1qb, C1qc), which are highly expressed within microglia under homeostatic conditions (**Figure 3**). Similarly, tyrobp and Trem2, which formed phagocytic synapses (48), are both highly expressed in microglia during myelin loss. Finally, a wide variety of upregulated gene transcripts are associated with MHC class II antigen presentation and modulation of T cell function. This includes H2-Ab1, encoding for the MHC class II molecules and H2-DM, encoding for a second accessory protein, which facilitates peptide loading. Similarly, genes associated with the invariant chain of MHC class II were increasingly expressed within microglia, such as Cd74 and Ctss (Cathepsin S, which cleaves invariant chain thereby promoting loading on MHC Class II). In addition, genes encoding for modulators of the CD4 T cell response, such as Itgax (CD11c), Cd86 and Cd83 were also expressed by microglia (**Figure 7B**). Overall, the upregulated networks are related to complement activation, enhanced class I and class 2 antigen processing and presentation (potentially related to IFN-γ responses) as well as migration and

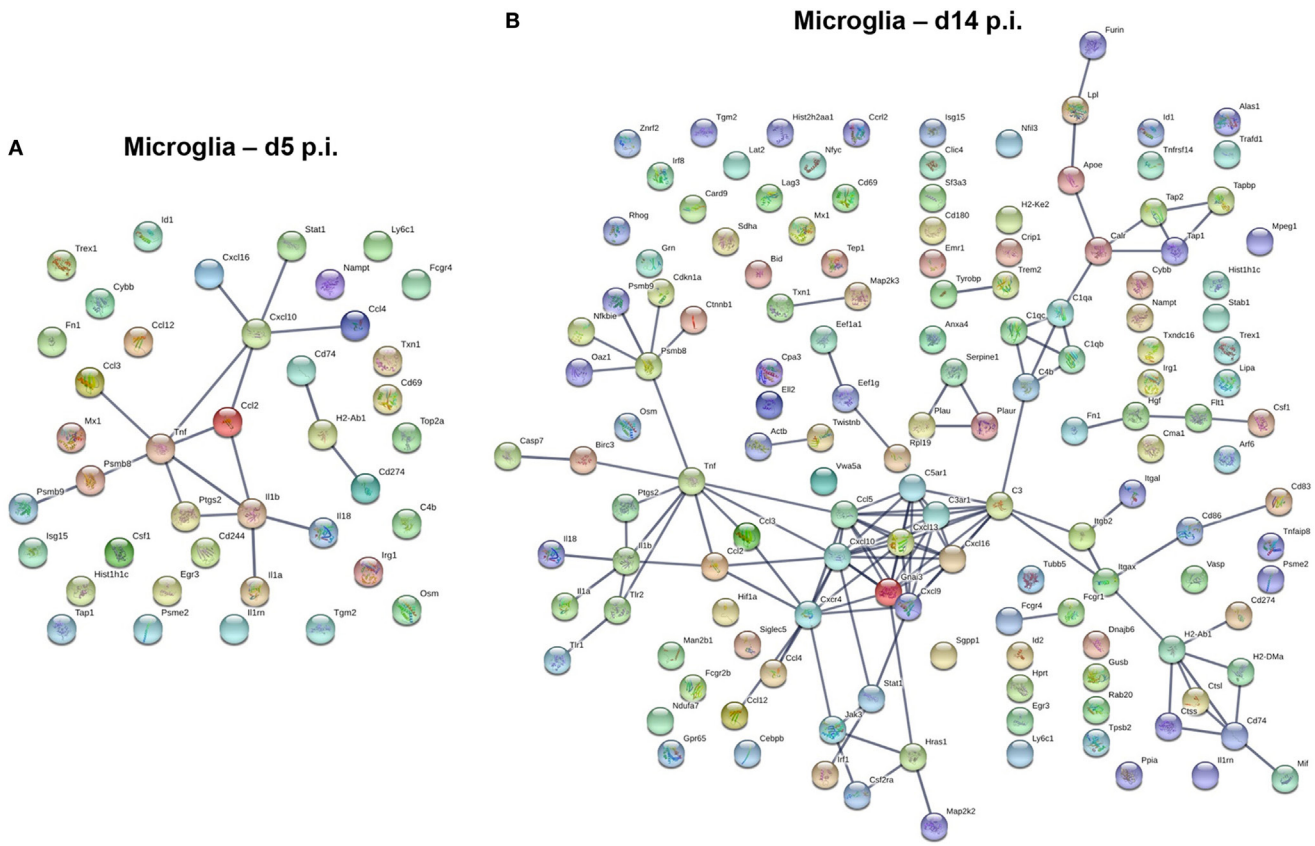

FIGURE 7 | Upregulated gene network within microglia at d5 p.i. and peak demyelination. Protein–protein network constructed based on microglia genes upregulated by at least twofold at days 5 **(A)** and 14 **(B)** p.i. compared to naïve levels were analyzed using Ingenuity IPA software.

phagocytic activity. However, there does not appear to be a bias toward phagocytic receptors over other components activated by pro-inflammatory mediators.

Microglia Repressed Anti-Inflammatory Genes During JHMV-Induced Demyelination

Pathogenic versus protective functions of myeloid cells following activation have also been correlated to expression of key molecules defined as M1 versus M2 markers. While the strict classification of myeloid cells into the M1 or M2 category has been tempered based on a more dynamic and mixed phenotype during inflammatory responses (49, 50), the M1 and M2 markers remain helpful to gage overall effector functions. Among the 89 analyzed gene transcripts in the Nanostring myeloid panel related to M1/M2 polarization (51 M1 and 38 M2 genes), between 50 and 59% were upregulated across the course of JHMV infection with no difference comparing M1 versus M2 genes (**Figure 8A**).

Among the total upregulated M1 markers, about 50% were commonly increased within both infiltrating BMDM and microglia; representative genes were Il12b and Il15Ra (**Figures 8A,B**). However, while high levels of Il12b mRNA were observed in both BMDM and microglia at d5 p.i., expression was only sustained in microglia at day 7 p.i. and thereafter (**Figure 8B**). By contrast, IL15ra was increased in both BMDM and to a lesser extent in microglia at d5 p.i., but was decreased in both populations at later time points p.i. (**Figure 8B**). In addition, between 35 and 45% of M1 markers were specifically expressed by infiltrating BMDM during the course of JHMV infection (**Figure 8A**), including Cd86, atf3, Ifng, Ptgs2, Ccr7, Cxcl11, and Cxcl12 transcripts (**Figure 8B** and data not shown). However, only 5 M1 related gene transcripts were specifically upregulated in microglia at the time of demyelination, including Ccl5, Fas, Cxcl13, Tnfsf10, and Psmb9. (**Figures 8A,B** and data not shown).

Importantly, the most prominent difference between microglia and BMDM was noted in M2 marker regulation. Among the 50–58% M2 gene transcripts upregulated following JHMV infection, only a small proportion (21–32%) was expressed by microglia (**Figure 8A**). Fn1 was the only M2 marker specifically expressed by microglia at the time of demyelination (**Figure 8A**). Although Il1rn transcript expression was elevated in both BMDM and microglia, the increase was at best modest in microglia (**Figure 8C**). The majority (86–95%) of M2 markers upregulated during JHMV infection were rather expressed by infiltrating BMDM, including Arg1, Erg2, Il-10, and Ccl22 (**Figures 8A,C** and data not shown). Increased transcript levels were most pronounces at days 5 and 7 p.i., but dropped off thereafter. Altogether,

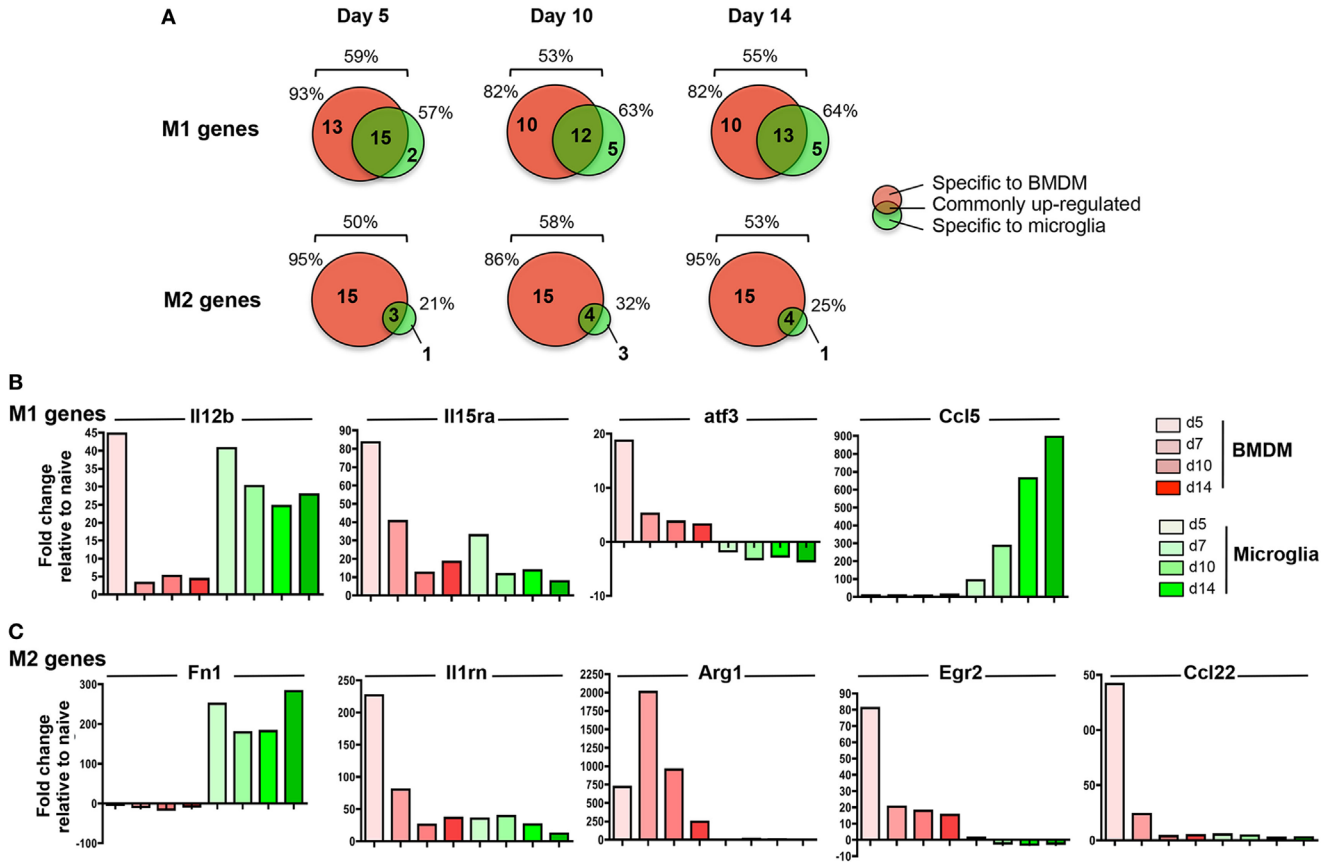

FIGURE 8 | M1 and M2 gene regulation following JHMV infection. bone marrow-derived macrophage (BMDM) and microglia gene expression patterns obtained from infected mice described in **Figure 4** were analyzed for **(A)** the number and distribution of M1 and M2 genes upregulated specifically in one or both populations at days 5, 10, and 14 p.i. Percentages above each diagram represent the overall proportion of M1 or M2 genes upregulated at each time point, while percentages on each side of the diagram represent the proportion of regulated genes with increased expression in BMDM versus microglia. The fold expression change of **(B)** select M1 genes (Il12b, Il15ra, atf3, Ccl5) and **(C)** select M2 genes (Fn1, Il1rn, Arg1, Egr2, Ccl22) compared to naïve levels within microglia and BMDM between days 5 and 14 p.i. is represented in bar graphs.

these data showed that while infiltrating BMDM express a mixed phenotype of M1 and M2 markers during JHMV infection, microglia expressed primarily pro-inflammatory genes while not expressing M2 markers.

DISCUSSION

Microglia and infiltrating macrophages are major components of MS active lesions (51). Their effector functions are highly heterogeneous as evidenced by both pathogenic and protective functions during the course of MS (7). They can promote tissue damage by releasing toxic and pro-inflammatory molecules, mediate demyelinated axons through phagocytosis as well as propagate inflammation by recruiting and activating adaptive immune cells. On the other hand, both populations also display protective functions by clearing myelin debris, which facilitates remyelination, as well as releasing trophic and anti-inflammatory factors, which promote tissue repair. While it remains a challenge to distinguish infiltrating macrophages from microglia in MS lesions due to morphological and phenotypic similarities, they are disparate effector cells based on animal MS models (17, 52). Questions relating to the pathogenicity of infiltrating macrophages and/or microglia in MS remain unanswered. Can both populations display protective functions? Do they display dynamic functions throughout the evolution of MS lesions? Deciphering the respective roles of macrophages versus microglia in facilitating tissue damage and/or repair is essential to our understanding of MS pathogenesis and development of effective therapeutic strategies.

In the murine EAE model of MS, infiltrating BMDM are essential in mediating demyelination (53). Gene expression profiles demonstrated that BMDM are indeed highly phagocytic and inflammatory at disease onset, while microglia display a repressed phenotype (17). By contrast, during JHMV-induced demyelination, recruited BMDM are dispensable for the demyelinating process (30). Distinct from EAE, where microglia activation precedes CNS infiltration of BMDM (52), JHMV infection elicits early BMDM infiltration, prior to microglia activation. These distinct kinetics of BMDM recruitment relative to microglia activation thus appear to correlate with the

apparently opposing roles of microglia as demyelinating populations. These data further suggest that early responses set the stage or imprint subsequent effector functions of BMDM and microglia. Using a similar approach with Nanostring analysis as in EAE, the present study used gene expression profiling to characterize both BMDM and microglia myeloid functions at various times post JHMV infection. Analysis of overall gene expression patterns revealed that the most extensive changes in BMDM were evident early after infection, while microglia showed a more dynamic profile throughout the course of viral encephalomyelitis. Importantly, the most drastic gene upregulation in microglia was observed coincident with demyelination, at which time peak viral load and T cell effector function have substantially subsided (54). Our data contrast with EAE (17), where BMDM upregulated far more genes compared to microglia at disease onset, supporting opposing functions of BMDM and microglia in mediating demyelination in these two models. Further, while BMDM exhibited a mixed expression profile of both pro- and anti-inflammatory markers, microglia expressed a highly pro-inflammatory profile and repressed most of the M2 markers across the entire time course of JHMV encephalomyelitis.

Analysis of protein-interacting networks within genes upregulated in microglia at the time of myelin loss revealed several key functions linked to promoting tissue damage. Genes associated with complement activation were notably increased, although they were already highly expressed by microglia under homeostatic conditions. Complement activation as a pathogenic component in MS has been reported following detection of deposits of the activated products of the complement component C3 in MS lesions (55). The classical complement pathway has also been shown to mediate OLG death thus promoting demyelination (56). Microglia phagocytic activity may also initiate tissue damage by directly removing myelin from axons, especially at the node of Ranvier (17). Genes associated with TREM2/DAP12 signaling were also highly expressed by microglia at time of demyelination. TREM2 modulates phagocytic capacity of myeloid cells *via* DAP12 signaling (57) and is expressed on myelin-loaded myeloid cells in MS lesions (58), supporting a role in MS pathogenesis. Similar to JHMV infection, TREM2 is predominantly expressed by microglia during EAE and cuprizone-induced demyelination (59–61). However, TREM2-modulated phagocytic functions are essential for removal of myelin debris and remyelination implicating repair-promoting functions of microglia in the specific tissue environments defining these two demyelination models (17, 62). Preferential TREM2 expression within microglia compared to BMDM following JHMV infection support a more pathogenic role of TREM-2 in JHMV-induced demyelination, potentially by promoting myelin stripping after recognition of glycolipids and phospholipids exposed on damaged myelin. In this context, it is critical to note that JHMV infection is associated with extensive transient production of IFN-γ and its inducible genes, i.e., iNOS, and CXCR3 ligands, which may drive a more phagocytic pathway in microglia in efforts to remove damaged proteins and lipids (54). Further investigation is required to define inflammatory conditions under which TREM-2 modulated or other phagocytic pathways promote tissue damage or repair and whether these are transient and reversible. Microglia may also induce demyelination by secreting toxic factors including inflammatory cytokines that are highly expressed by microglia at time of demyelination, including TNF. TNF can induce OLG death (63, 64) and is expressed in active MS lesions, as well as elevated TNF in serum and cerebral spinal fluid correlates with enhanced MS pathology (65, 66). Finally, microglia functions during JHMV infection were also associated with promoting adaptive immune response. An extensive network of chemokines and chemokines receptors relating to the recruitment and arrest of T and B cells within the inflamed CNS were highly expressed by microglia. Similarly, several genes associated with antigen processing and presentation by MHC class I and II molecules were upregulated within microglia, suggesting that microglia promote T cell reactivation upon CNS entry. However, microglia explanted during EAE, TMEV as well as JHMV infections failed to support myelin-specific CD4 T cell responses *ex vivo*, despite detection of internalized myelin (67–69). A potential deficit in antigen processing was supported by the ability of exogenous peptide to overcome the inability of microglia to prime myelin-specific CD4 T cells (69). Nevertheless, this notion is opposed by our microglia profiling showing upregulation of genes involved in protein degradation and class II peptide loading, e.g. CD74 (Invariant chain), H2-DMa (peptide loading), Ctss (Cathepsin S), which cleaves invariant chain. The apparent inability of microglia to elicit CD4 T cell effector function *ex vivo* thus remains intriguing.

Our present study reveals new insights into the plasticity of microglia in adapting to inflammation and expressing pathogenic functions associated with demyelination, characteristics which have previously been ascribed to BMDM (17). Moreover, altered BMDM expression profiles coincided with their early infiltration into the CNS and remained largely similar throughout infection. While altered microglia gene expression coincided with the time of early, yet robust demyelination, it remains to be determined whether these changes are sustained at later time points during JHMV persistence, when clinical disease improves and remyelination occurs. It will be of specific interest to assess whether the microglia pro-inflammatory phenotype evolves to an anti-inflammatory, repair-promoting phenotype, as evidenced by the plasticity of myeloid cells in CNS autoimmunity (70). Furthermore, our study emphasizes that the distinct tissue environments during EAE and JHMV infection drive opposite effector functions of microglia versus infiltrating macrophages. The interplay between T cells, infiltrating macrophages and microglia, as well as astrocytes drives MS pathogenesis, yet mechanisms ultimately leading to loss of repair remain unclear. Taking advantage of demyelinating models characterized by distinct inflammatory factors such as both Th1 and Th17 cells in EAE (11), strong Th1 polarized responses during JHMV infection, distinct kinetics of BMDM recruitment versus glia activation promises to reveal essential new insights into the interplay of microglia and BMDM functions in debris clearance versus active myelin stripping and ongoing axonal damage. Longitudinal studies will aid in developing efficient future therapies to combat MS pathogenesis.

ETHICS STATEMENT

All procedures were performed in compliance with protocols approved by the Cleveland Clinic Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

CS designed and performed experiments, collected and interpreted the data, and wrote the manuscript. RD analyzed data and edited manuscript. CB designed the research, provided materials, interpreted the data, and wrote the manuscript. All authors approved the final 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.2018.01325/full#supplementary-material.

FIGURE S1 | Validation of Nanostring nCounter gene expression analysis by real-time PCR. To validate Nanostring nCounter data, expression of IL-15, Il1Rn, and Arg1 was analyzed by Q-PCR in naïve circulating monocytes and microglia, as well as in bone marrow-derived macrophage (BMDM) and microglia isolated from the spinal cord of JHMV-infected mice at days 5, 7, 10, and 14 p.i. Levels of mRNA expression are normalized to GAPDH mRNA levels.

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REVIEW
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The Kaleidoscope of Microglial Phenotypes

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Gene expression analyses of microglia, the tissue-resident macrophages of the central nervous system (CNS), led to the identification of homeostatic as well as neurological disease-specific gene signatures of microglial phenotypes. Upon alterations in the neural microenvironment, either caused by local insults from within the CNS (during neurodegenerative diseases) or by macroenvironmental incidents, such as social stress, microglia can switch phenotypes—generally referred to as "microglial activation." The interplay between the microenvironment and its influence on microglial phenotypes, regulated by (epi)genetic mechanisms, can be imagined as the different colorful crystal formations (microglial phenotypes) that change upon rotation (microenvironmental changes) of a kaleidoscope. In this review, we will discuss microglial phenotypes in relation to neurodevelopment, homeostasis, *in vitro* conditions, aging, and neurodegenerative diseases based on transcriptome studies. By overlaying these disease-specific microglial signatures, recent publications have identified a specific set of genes that is differentially expressed in all investigated diseases, called a microglial core gene signature with multiple diseases. We will conclude this review with a discussion about the complexity of this microglial core gene signature associated with multiple diseases.

Keywords: microglia, activation, phenotype, transcriptome, neurodegenerative diseases, core gene signature

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INTRODUCTION

Macrophages are innate immune cells that reside in all organs of the body. They have versatile functions that are tailored to the organ of residence (1). Genome-wide studies showed that microenvironment-specific signals establish tissue-specific properties of macrophages *via* epigenetic mechanisms (2, 3).

Transcriptomic analyses are an effective way to determine gene expression patterns that serve as a proxy for different cellular states under different conditions. The last decade, numerous transcriptome studies of (micro)glia have been published and provide much insight in glia biology (4, 5).

Gene expression profiling of purified microglia has confirmed that they are central nervous system (CNS)-resident macrophages that express many genes typical for the myeloid lineage, including receptors for pathogen-associated molecular patterns and damage-associated molecular patterns, genes involved in phagocytosis and antigen presentation. This makes distinction between microglia and macrophages, particularly under neuropathological circumstances, very difficult (6). A common approach to separate microglia from other cells of the periphery and CNS is the preparation of a single-cell suspension followed by fluorescence-activated cell sorting (FACS) based on the membrane expression of $CD11b^{high}$ and $CD45^{low/int}$ in mice and human (7). In mice, Ly6C/Ccr2 and Mrc1 are specifically expressed by monocytes (8) and CNS interface macrophages (9), respectively and can be additionally used to distinguish between these cells

and microglia. In humans, although not yet widely applied, CCR2 and CD14 are used to discriminate between microglia and monocytes (10).

In recent years, RNA expression profiling of microglia has received much attention (see the glia open access database) (11). This review will focus on microglial phenotypes in the CNS related to manifold processes associated with brain development, physiology, and pathology.

MICROGLIA ORIGIN AND HOMEOSTASIS

Microglia Ontogeny

As already proposed by Río-Hortega in 1919 (12), sophisticated *in vivo* lineage tracing studies confirmed the mesodermal origin of microglia during embryogenesis (13–15). This is different from other CNS cells that arise from the neuro-ectoderm (16). Even within the mesoderm-originating myeloid cell compartment, microglia have a distinct ontogeny. In mice, tissue-resident macrophages emerge from two waves of erythromyeloid progenitor (EMP) production (primitive and transient definitive hematopoiesis) in the extra-embryonic yolk sac (YS) prior to the establishment of definitive hematopoiesis in the fetal liver and later in adult bone marrow (14, 17). Microglia originate from the primitive hematopoietic wave of early EMP's (primitive macrophages) at embryonic day 7.5 (E7.5) in the YS—a process dependent on the transcription factors (TFs) *Spf1* (*Pu.1*) and *Irf8* (14, 15).

These primitive macrophages spread *via* the bloodstream to the developing organs, including the neurepithelium, which gets colonized by primitive macrophages (microglia) as early as E9.5 (13). By contrast, other tissue-resident macrophages mainly develop from the transient definitive hematopoietic wave of late EMP's arising at E8.5 in the YS. These late EMP's subsequently colonize the fetal liver from E10 onward and mature into tissue macrophages *via* a monocytic intermediate (14, 17). Currently, it is not yet resolved why these differences in microglia and macrophage ontogenies exist.

Although human microglia ontogeny is not yet studied in such detail, immunostaining of the human encephalon indicates the presence of IBA1 positive microglia at gestational week 5.5, that enter the brain *via* the ventricles. Microglia proliferate and develop toward their typical ramified morphology from that time point onward (18). However, the ontogeny of human microglia remains to be defined in detail.

Microglia Development Occurs in Four Consecutive Stages

A recent study combining transcriptome with epigenome analysis identified genes and chromatin modulators that regulate different stages of microglia development in mice (19). Microglial gene expression clusters are identified that are specific for four sequential developmental phases: YS (E10–12.5), early microglia (E10.5–14), pre-microglia [E14—postnatal day 9 (P9)], and adult (P28 onward). Early, pre- and adult microglia are marked by genes related to cell cycle and proliferation (*Dab2*, *Mcm5*, and *Lyz2*), synapse pruning (*Crybb1*, *Csf1*, and *Cxcr2*), and immune surveillance (*Mafb*, *Cd14*, and *Mef2a*), respectively. These developmental stage-specific gene ontology (GO) terms (unifying terms annotating a global function to genes) match with typical microglia functions, including the involvement in neuronal network refinement (synapse pruning) (20, 21) and maintenance of adult brain homeostasis (22). Extensive parallel single-cell sequencing of microglia identified a high degree of homogeneity of microglia populations at specific developmental stages. Concordantly, the expression level of developmental stage-specific genes correlate to the accessibility of corresponding enhancers identified by dimethylation of lysine 4 on histone 3 (H3K4me2)-enriched regions distal from the transcription start sites of a gene. Whereas YS and embryonic microglia cluster more closely together at the transcriptional level, embryonic and pre-microglia cluster more closely together at the epigenetic level. These results indicate that the microenvironment is driving gene expression through modulation of the epigenetic landscape into a permissive state for the expression of gene patterns belonging to specific developmental phases. This suggestion is corroborated by the fact that environmental perturbations, such as in germ free (GF) mice and maternal immune activation, led to abnormal microgliosis. Mice that are subjected to maternal immune activation display a shift from the pre-microglia stage toward a more advanced developmental stage, due to a decreased expression level of inflammatory and defense related genes. It is hypothesized that the disruption of microglia development disturbs physiological microglial functions (19).

In addition, specific potential TF binding motifs are identified in promotor regions of genes specifically expressed at different microglial developmental phases. Clearly, the previously identified TFs *Pu.1* and *Irf8* are essential for microglia development (15) and are highly upregulated throughout microglia development (19). These results corroborate the finding that *Pu.1* is essential for the gene regulation of several functions including myeloid cell differentiation, chemotaxis, and phagocytosis (23–25). In line with the findings of *Irf8* in the study of Kierdorf and coworkers (15), *Irf8* is also important in myeloid cell lineage differentiation and survival during early hematopoiesis in zebrafish (26).

The TF *Mafb* is enriched in adult microglia and the identification of immune and viral GO terms, enriched in the pre- and adult microglia signature of *Mafb*-deficient microglia, suggests that *Mafb* is crucial for the regulation of brain homeostasis.

In a similar approach, consistent findings according to microglial developmental stages and stage-specific functions were recently identified (27). Interestingly, it was shown that microglia progenitors in the YS and at E10.5 already express a part of the homeostatic microglial signature genes (see The Homeostatic Microglial Gene Signature in Mice and Men), which then expand with increasing developmental stage. Based on genes expressed at all developmental stages, a murine microglia development signature containing 568 genes was identified and compared to gene expression data of FACS-purified microglia from human fetuses ranging from 14 to 24 weeks of estimated gestational age. This analysis identified 387 overlapping genes, involved in functions as immune response and phagocytosis. In addition, it was shown that microglia derived from E16.5

mice are developmentally corresponding to microglia derived from mid-trimester human fetuses (14–24 weeks of pregnancy). Although these human microglia already express genes belonging to the homeostatic microglial gene signature, it should be of note that human fetal microglia do not (yet) seem to be sexually dimorphic (see Microglial Sexual Dimorphism) (27).

Of importance, it seems that deviations in the microglial developmental transcriptome are linked to the development of neurological diseases, such as autism and Alzheimer's disease (AD) in adulthood (28).

In conclusion, microglial development occurs in a complex and fine-tuned sequence of processes regulated by environmental signals and is associated with specific gene expression programs.

The Homeostatic Microglial Gene Signature in Mice and Men

Over the last years, the transcriptome of homeostatic murine microglia was identified (2, 3, 29–33). Under homeostatic conditions, this transcriptome contains genes specifically expressed by microglia in comparison to other CNS cells and myeloid cells, hereafter referred to as homeostatic microglial signature genes. These genes are now widely used by other researchers to identify and study microglia, also under disease conditions (see **Box 1**).

The Homeostatic Gene Signature of Murine Microglia

The first gene expression profile of murine microglia was obtained in 2012 in a microarray study (32). Based on these expression data, that included a common macrophage signature, several gene clusters were identified. However, distinct gene expression signatures among four different macrophage populations were identified. 64 genes, including *SiglecH* and *Cx3cr1* were shown to be more abundantly expressed in microglia when compared to other investigated macrophage types. Chiu et al. identified 29 genes that are highly specific for microglia, including *Olfml3*, *Tmem119*, *and SiglecH* (31).

Direct RNA sequencing revealed the microglial sensome, consisting of 100 cell surface receptors and proteins specific for the sensing of microenvironmental factors, including pattern recognition-, chemokine-, Fc-, purinergic-, cytokine-, extracellular matrix-, and cell–cell interaction receptors (33). Approximately half of these genes seem to be regulated by Tyrobp (Dap12), a protein tyrosine kinase binding protein and ligand for Trem2, both belonging to the homeostatic microglial signature genes (33). The Trem2–Dap12 signaling pathway seems to be involved in (1) the suppression of toll-like receptor (TLR)-induced inflammation, (2) mediating phagocytosis, and (3) reduction of cell death and enhancement of myeloid cell proliferation (42). Analogous to other studies, Hickman and coworkers identified several genes that are shared by microglia and other tissue macrophages, but also macrophage subtype-specific expression of gene sets. The top 25% uniquely expressed genes in microglia contain many of the sensome-including genes (33).

In 2014, two studies were published that extensively investigated microglia and other tissue-resident macrophages at transcriptome level (2, 3). These studies also address the epigenetic differences between different macrophage subsets, observing a positive correlation with the transcriptome. When comparing large peritoneal macrophages (LPM), small peritoneal macrophages (SPM) and microglia at the transcriptional level, both macrophages and microglia are depended on *Pu.1*. However, the co-enrichment of different motifs was revealed, LPM and SPM are thus shown to be depended on retinoic acid (RA) receptors (RAR α/β) whereas motifs as SMAD, consistent with the TGFβ signaling in the brain is shown to be unique for microglia (2). Analysis of seven different tissue-resident macrophage populations identified 3.348 differentially expressed genes. K-means clustering of these genes led to the discovery that the microglial cluster (consisting of 641 genes that are higher expressed in microglia) is different from other tissue macrophages, where *Sall1* is found to be most highly expressed in microglia (3).

A Tgf-β-dependent homeostatic microglial gene signature consisting of 152 unique microglial genes, *P2ry12*, *Tmem119*, *Fcrls*, and three microRNAs (miRNAs) is identified by comparing the microglial transcription profile to that of other CNS cells and monocytes. The validity of these genes being uniquely expressed in microglia is confirmed by mass spectrometry, since many of these genes are also detected in the enriched fraction of microglial proteins. It is shown that Tgf-β is a crucial factor for the establishment of the microglial homeostatic gene signature, since mice that endogenously lack Tgf-β in CNS tissue show a remarkable reduction in microglial numbers and the remaining microglia show significantly reduced expression of these homeostatic microglial signature genes (30).

A transcriptome profile of isolated microglia that closely approximates their *in vivo* status was published by Bennett and coworkers, using a relatively non-invasive method to purify microglia (29). Inflammation-associated genes (*Il1b*, *Nfkb2*, and *Tnf*) are significantly lower expressed in this dataset compared to others (2, 31, 32), indicating that *in vitro* procedures influence the homeostatic microglial gene signature (see The Transcriptome

BOX 1 | Environmental influence on the microglial homeostatic gene signature.

Evidence is accumulating that genes belonging to the homeostatic microglial gene signature are downregulated during neurological diseases. It was shown that microglia uniformly downregulate their homeostatic signature genes, such as *Sall1*, *Pu.1*, *Tmem119*, *Cx3cr1*, and *P2ry12/13* and upregulate risk genes for AD, such as *Apoe* and *Trem2*, in mouse models for aging, AD (5XFAD and APP-PS1), and amyotrophic lateral sclerosis (ALS) (SOD1) (34–36). Interestingly, homeostatic microglial signature genes seem to be differentially expressed at different disease stadia in EAE, a mouse model for multiple sclerosis (MS). A downregulation of homeostatic microglial signature genes is observed in acute and chronic EAE, whereas during the recovery phase of EAE, gene levels are restored to those of homeostatic microglia (34, 37). Concordantly, a loss of homeostatic microglial signature genes is identified in human MS brain tissue (38). Furthermore, homeostatic microglial signature genes seem to be at least partially downregulated during aging (33, 39, 40) and are differentially expressed in male and female murine microglia (27).

In addition, it was shown that microglia upregulate CD45 expression under different disease conditions (8, 41). Furthermore, monocytes downregulate Ly6C and Ccr2 during their differentiation into macrophages after infiltrating brain tissue (6, 8), resulting in issues regarding the distinction of microglia and peripheral monocyte/macrophages in disease conditions.

Concordantly, the use of specific markers to identify microglia under specific disease conditions is still controversial.

of *In Vitro* and *Ex Vivo* Microglia Is Different). Tmem119 was studied in detail and was identified to be a specific and, at least at protein level, robust microglial marker in mice and human, also under inflammatory/disease conditions. In addition, potential novel microglial functions associated with vascular development (*Pdgfb*), oligodendrocyte development (*Pdgfa*), and synapse formation (*Sparc*) are identified and microglial involvement in different neurological diseases (*Comt*, *Hprt*, and *Trem2*) are confirmed by the enrichment of the indicated genes in microglia (29).

The common denominator of at least these seven studies is the identification of the homeostatic microglial signature genes, including *Sall1*, *Hexb*, *Fcrls*, *Gpr43*, *Cx3cr1*, *Tmem119*, *Trem2*, *P2ry12*, *Mertk*, *Pros1*, and *SiglecH*, that are uniquely/higher expressed in microglia and not or only at low levels in other brain cells or myeloid cell types, including tissue-resident macrophage subsets and monocytes.

The Homeostatic Gene Signature of Human Microglia

In parallel to the identification of homeostatic microglial signature genes in mice, two studies identified homeostatic gene signatures of human microglia. Gosselin and coworkers investigated the transcriptomes of microglia purified from healthy-appearing brain tissue obtained during neurosurgery of 19 young patients (0–17 years) with epilepsy, tumors, or acute stroke. The top 30 highly expressed genes in that dataset are related to functions like microglial ramification and motility (*P2RY12* and *CX3CR1*), synaptic remodeling (*C3* and *C1QA-C*), and immune response (*HLA-DRA* and *HLA-B*). The comparison of microglia-specific and whole cortex gene expression profiles identified 881 homeostatic human microglial signature genes, including *CX3CR1*, *P2RY12*, and several complement factors as *C3*, *C1QA*, *C1QB*, and *C1QC*. In addition, these human microglial homeostatic signature genes significantly overlap with transcriptomic datasets related to different neurological diseases, including AD and Parkinson's disease (PD), in which many of the human homeostatic microglial signature genes are differentially expressed, indicating an important role of microglia in the pathophysiology of these diseases (43).

Another study identified the homeostatic human microglial gene signature from a population of 39 adult (34–102 years) postmortem donors in The Netherlands and Brazil. This homeostatic microglial gene signature is characterized by 1,297 genes that are significantly differentially expressed in purified microglia when compared to whole parietal cortex cell lysates. GO analysis indicated that these genes are related to the innate immune system, including functions as pathogen and self-recognition, inflammasome, cell adhesion and motility (C3XCR1), immune signaling and modulation (*P2RY12*, *Q1QA-C*, and *HLA-DR*). In addition, risk genes for neurodegenerative diseases, such as *APOE* and *TREM2* are enriched in purified adult human microglia (39). In addition, the two TFs *PU.1* (*SPI.1*) and *IRF8*, which are also crucial during murine microglia ontogeny and development (19) are highly expressed in both datasets (39, 43).

Thus, together these two studies identified the homeostatic human microglial gene signature, that shares many genes with the murine homeostatic gene signature, but also seem to possess human-specific properties.

The homeostatic microglial gene signature is conserved across species (39, 43). Comparison of the two homeostatic human microglial gene signatures to several homeostatic murine microglial gene signatures reveals an overlap of more than 50%, depending on the specific datasets that were compared (39, 43). The genes *APOC1*, *MPZL1*, *SORL1*, *CD58*, *ERAP2*, *GNLY*, and *S100A12*, most closely related to the innate immune system, are specifically found to be expressed in human microglia and not, or only to a very low extent in murine microglia. Concordant with the high overlap between murine and human transcriptomes are the identified similar epigenetic landscapes, i.e., identified microglial-specific regulatory regions, in murine and human microglia (43). Concluding, research of recent years has identified the homeostatic murine and human microglial gene signatures, which enables better identification and investigation of microglia in murine and human tissue.

Microglial Sexual Dimorphism

Sex-specific transcriptomic signatures are found when comparing adult male and female mice. A higher gene expression level of inflammatory response genes, such as *Ccl2*, *Tnf*, *Irf1*, *Cxcl10, and Il1b* is found in female mice, indicating a more immune-activated state of female microglia. In addition, homeostatic microglial signature genes are differentially expressed in male and female mouse microglia. Interestingly, it is demonstrated that environmental alterations during embryogenesis, like the absence of the maternal microbiome (GF mice), has different effects on male and female microglial transcriptomes at the identified developmental stages (see Microglia Development occurs in Four Consecutive Stages). Whereas the transcriptome of microglia from GF offspring does not seem to be overtly altered at E14.5 when compared to control microglia (under specific pathogen-free conditions), it is affected at E18.5 especially in males and in adults especially in females, characterized by 1,216 and 433 differentially expressed genes, respectively. From those 1,216 differentially expressed genes in GF E18.5 males, the majority is downregulated and involved in functions such as translation, endocytosis, and metabolism. Regarding the 433 differentially expressed genes in GF adult female microglia, approximately half of these genes are downregulated and involved in the inflammatory response, whereas the upregulated genes are associated with the regulation of transcription. In addition to transcriptomic changes, the pattern of microglial colonization into the neocortex also occurs in a sex-dependent manner in offspring from GF dams. Whereas male offspring of GF dams show an increased microglial density prenatally (E18.5), female offspring of GF dams show an increased microglial density postnatally (P20) (27).

Concordant with the findings by Thion and coworkers a developmentally more mature state, marked by upregulation of genes involved in immune processes, is identified in female microglia compared to male microglia at P60. This result is based on the microglial developmental index (MDI) that is calculated by the ratio of the average expression of globally upregulated genes divided by the average expression of globally downregulated genes in a developmental time course from E18 to P90 in male and female mice (28). Upon lipopolysaccharide (LPS) treatment, the MDI of male microglia increases, indicating maturation of

male microglia in response to LPS. These sex-dependent baseline and LPS-induced changes in the transcriptome are accompanied by sex-specific microglial morphologies in the adult hippocampus of mice. When compared to the morphology of baseline female microglia, baseline male microglial morphology seems to be more complex. It is marked morphologically by an increased process volume and area and an increased number of branches and intersections, albeit female and male differences are only statistically significant for the parameter process volume. Upon LPS stimulation, baseline morphological characteristics of male microglia get significantly reduced, whereas those of female microglia do not change much (28).

Concluding, murine microglia seem to respond to environmental insults in a sex-dependent manner, which was not yet manifested in human microglia (27).

Microglia Possess Brain Region-Specific Transcription Profiles

Insight in regional heterogeneity of microglial phenotypes can provide necessary information on regional specific microglial functions. RNA sequencing of bulk samples containing large numbers of microglia from the whole brain might mask specific regional heterogeneity. Whereas microglia are important for general functions including scanning the microenvironment, phagocytosis, and neuronal support (22), specific, additional regional microglial functions could be envisioned.

Several mouse brain regions were compared, exploring the hypothesis that the microenvironment could shape microglial functions (40). Regional transcriptional heterogeneity is observed when microglia from the mouse cerebral cortex, hippocampus, cerebellum, and striatum are compared. Three transcriptomic clusters are identified, specific for cerebral cortex/striatum, hippocampus, and cerebellum. Annotation of associated biological processes revealed that the hippocampal microglial gene cluster is involved in energy production and regulation, whereas the cerebellar and cortical clusters are associated with genes involved in immune response and regulation. Concordantly, TF binding motif analysis found TFs regulating the expression of bioenergetic genes and immune and inflammatory genes to be over-represented in the hippocampal- and the cerebellar cluster, respectively. Interestingly, there seems to be a difference in the immune-activation state of microglia belonging to the cortical and the cerebellar cluster. Cortical microglia show an increased expression of genes coding for inhibitory immunoreceptors, including *Trem2* and *SiglecH*, whereas cerebellar microglia show an upregulation of genes coding for activating immunoreceptors, indicating a more immune-activated microglial phenotype, different from the LPS or IL-43-induced microglial phenotypes. Notably, approximately one-third of the microglial sensome genes (belonging to the homeostatic microglial gene signature) are differentially expressed in microglia derived from different brain regions. Concluding, although microglia from different brain regions share the expression of specific genes, they also express region-specific gene sets indicating region-specific microglial functions (40).

De Biase and coworkers reported different microglial phenotypes when comparing regions in the basal ganglia. The transcriptome of ventral tegmental area (VTA) microglia appears to be most distinct when compared to microglia in the nucleus accumbens and substantia nigra pars compacta (SNc). Differentially expressed genes in microglia of the VTA are involved in metabolic processes such as mitochondrial function, glycolysis, gluconeogenesis, and oxidative phosphorylation. Microglia in the VTA and SNc show limited surveillance and contribution in homeostasis, based on observations made in cell density, branching, and lysosome content. Based on the overlapping microglial genes in the different regions, classical microglial cell functions are preserved among the different regions. However, microglia in different regions also exhibit regional adaptation (44), a finding consistent with that of Grabert and coworkers.

In another study, microglia were compared with non-parenchymal CNS macrophages in the subdural meninges, perivascular spaces, and the choroid plexus on single-cell transcriptome level. Gene expression profiles of microglia and the three investigated CNS interface macrophage populations display high similarity in contrast to peripheral monocytes. When compared to the monocytic transcriptome, microglia and non-parenchymal macrophages share 443 differentially expressed genes, such as abundant expression of the myeloid markers Cx3cr1, Csfr1, and *Aif*(*Iba1*). The high overlap of transcriptomes between these brain-associated macrophages might be based on their similar ontogeny and kinetics, since perivascular and meningeal macrophages, analogous to microglia, also arise during primitive hematopoiesis in the YS and are long-lived cells that do not get replenished by peripheral monocytes. Besides this commonly expressed gene set, microglia and non-parenchymal macrophages also express unique separate gene sets. Microglia show differential expression of 2,328 unique genes that are unaltered in expression in non-parenchymal macrophages after comparison to the monocytic transcriptome. As an example, *P2ry12* and *Mrc1* are enriched in microglia and perivascular macrophages, respectively, and thus are used to distinguish these brain-associated macrophage populations (9).

Although not studied such extensively, it seems that human microglia also show brain region-specific gene expression profiles (45).

Concluding, the CNS is populated by different macrophage cell types, and even microglia in the parenchyma can be subdivided into different phenotypes based on their gene expression profiles, which might be associated with specific functions.

The Lifetime of Microglia

Microglia, as well as other tissue-resident macrophages (46, 47), are stable, self-renewing cell populations over the entire lifespan of an animal. The self-renewing capacity of microglia has been shown in an experiment where microglia were ablated using the Cx3Cr1^{CreER}:iDTR system. Within 5 days the 20% remaining microglia completely repopulated the CNS (48). This process was independent of infiltration of peripheral monocytes but was dependent on microglial interleukin-1 signaling. In a similar experiment, where treatment with a macrophage Csf1r inhibitor caused ablation of 99% of the resistant microglia, a full repopulation of microglia *via* nestin-positive progenitors within

1 week after treatment was observed (49). While it is well accepted that, at least under physiological conditions, microglia are not replenished by peripheral macrophages, the lifetime of microglia is still a matter of debate. Askew and coworkers (50) reported that microglia are rather fast proliferating cells with a turnover rate of approximately 3 months. By contrast, Füger and coworkers and Tay and coworkers propose cortical microglia to be long-lived cells with turnover rates between 15 and 41 months, respectively (51, 52). Turnover rates of microglia seem to vary between brain regions (50–52).

Although studying the lifetime of microglia in humans comes along with experimental limitations, estimations of human microglia turnover rates was made. It was estimated that the human microglia population might renew several 100 times within the average human lifetime of 80 years (50). By contrast, a relatively slow mean microglial turnover rate of approximately 28% per year and an average microglial age of 4.2 years is calculated using thymidine analog IdU (5-iodo-2′-deoxyuridine) labeling in brains of cancer patients and retrospective atmospheric ^{14}C measurements in the DNA of the same and healthy tissue postmortem (53). Clearly, the different microglial turnover rates that have been reported may be caused by the use of different methodologies and these findings need to be reconciled in the future.

Under disease conditions, it has been observed that microglia can transiently be replenished by monocyte-derived macrophages from the periphery, especially when the blood–brain barrier is disrupted (54, 55). In addition, turnover rates of microglia are increased under neurodegenerative conditions such as in the APP/PS1 mouse model for AD [containing AD risk mutations in the genes encoding for the amyloid beta precursor protein (*App*) and presenilin (*Psen1/Psen2*)] (51), unilateral facial nerve axotomy (FNX) in mice (52), and nitroreductase (NTR)-induced neurodegeneration in zebrafish larvae (55). Interestingly, during the resolution phase of neuroinflammation and -degeneration there seems to be a self-regulating mechanism returning microglial numbers in the CNS to physiological conditions. Intravital and electron microscopy of zebrafish larvae brains 8 days after NTR-induced neurodegeneration has shown that phagocytes (microglia and peripheral macrophages) either leave the CNS tissue with unknown destination or undergo apoptosis and are phagocytosed by viable microglia leading to a physiological microglia density in the forebrain numbers resembling those of healthy zebrafish (55).

In addition, microglial migration into regions distal to the neurodegenerative center as well as microglial apoptosis may contribute to the re-establishment of a homeostatic-like microglia density in the mouse brain during the resolution phase of unilateral FNX-induced neurodegeneration. The increased phagocytic activity of microglia identified by confocal microscopy as well as RNA sequencing during later phases of neurodegenerative resolution, led to the hypothesis that also in mammalian species microglia self-regulate their density by phagocytosing excessive microglia that have undergone apoptosis. After the resolution of neurodegeneration, a mixture of microglia that already existed and newly proliferated microglia is preserved (52).

Currently, it is not yet known whether a replicated cell is biologically younger or if it inherits (epi)genetic marks from the mother cell (51). It was shown that LPS treatment during embryonic development results in a dampened immune response (LPS tolerance) in the same mice when they are young adults (56), indicating that deviations in early microglial development have long-lasting effects on the microglial phenotype during aging and associated diseases.

THE EFFECT OF *IN VITRO* CONDITIONS ON THE HOMEOSTATIC MICROGLIAL GENE SIGNATURE

Human iPSC (hiPSC)-Derived Microglia-Like Cells

Restricted numbers of human microglia can be obtained by purification from human postmortem and surgically resected brain tissue. Unfortunately, limited access to viable human brain tissue causes a challenge to study human microglia and their respective roles in neurobiological diseases. One possibility to study human microglia on a relatively large-scale is the recent development that microglia-like cells can be generated from induced pluripotent stem cells (iPSCs). Thus, various studies describe protocols for the differentiation of hiPSCs into microglia-like cells. These differentiation protocols recapitulate microglial embryonic development and, in some cases, physiologic brain microenvironment *in vitro* (57–61). The first report, published by Muffat and coworkers shows differentiation of both human embryonic stem cells and hiPSCs into early YS myeloid-like cells and subsequently into mature microglia-like cells within 74 days (60). Douvaras and coworkers published a protocol that induces myeloid differentiation of hiPSCs into $CD14^+/CX3CR1^+$ microglial progenitors which mature under IL-34 and granulocyte macrophage colony-stimulating factor (GM-CSF) conditions into microglia in 60 days (58). Two other protocols describe the induction of a two-step differentiation process from hiPSCs into hematopoietic progenitor-like $CD34/CD43/CD45^-$ positive cells into microglia-like cells (57, 61). Haenseler and coworkers developed a protocol which induces the differentiation of hiPSCs into embryoid bodies followed by embryonic macrophage precursors in an M-CSF-, IL-3-dependent manner. Co-culturing of these hiPSC-derived macrophage precursors and cortical neurons in the presence of IL-34 and GM-CSF yields microglia-like cells within 14 days (59).

Human iPSC-derived microglia-like cells show several features of microglia *in vivo*: an amoeboid as well as a ramified microglia morphology, expression of microglial markers *IBA1*, *CX3CR1*, *CD11b*, *CD45*, and typical microglial functions such as phagocytosis, process motility, secretion of cytokines in response to LPS, IL-1β, or INF-γ stimulation, release of intracellular Ca^{2+} in response to adenosine diphosphate (ADP) and cell migration toward ADP and into 3D brain organoids (57–61).

To summarize, currently several protocols exist to obtain iPSC-derived microglial cells that partially approximate *in vivo* microglia.

The Transcriptome of *In Vitro* and *Ex Vivo* Microglia Is Different

Although hiPSC-derived microglia display specific *in vivo* microglial functions and express a proportion of the homeostatic microglial gene signature (57–61), gene expression profiles of hiPSC-derived microglia-like cells do not fully match those of *ex vivo* human fetal and adult microglia. Principal component analysis (57, 59, 60) and hierarchical clustering (58) of gene expression profiles revealed that hiPSC-derived microglia-like cells are most similar to cultured primary human microglia (57–60), and much less resemble non-cultured *ex vivo* human fetal microglia (61). This finding is confirmed when the transcriptome of murine embryonic stem cell-derived microglia is compared to the transcriptomes of *ex vivo* and *in vitro* murine microglia (62). Thus, the *in vitro* environment strongly determines their gene expression profile, leading to a microglial phenotype different from *ex vivo* microglia.

More specific analysis of *in vitro* cultured murine and human microglia revealed a significant upregulation of inflammatory and stress-related genes (30, 43, 63). Cultured microglia show repression of homeostatic microglial signature genes (2, 30, 43, 63, 64) and genes associated with microglial development (43, 63) already after 6 h in culture (43). In addition, *in vitro* conditions cause a reduced expression of microglial genes associated with different neurodegenerative diseases, e.g., *TREM2*, in human microglia (43). The *in vitro*-induced transcriptomic changes are mirrored by the observation of a remodeled epigenetic landscape of *in vitro* microglia. Microglia that were cultured for 7 days lost more than 50% of the super-enhancers identified in *ex vivo* microglia, including the *SALL1* super-enhancer (43), that regulates microglia identity and function (65). A decrease in H3K27Ac levels (associated with active transcription) is observed at accessible (ATAC-enriched) regions for microglial TFs, indicating an *in vitro*-induced loss of microglial (super-) enhancer activity. This finding is corresponding with the downregulation of *ex vivo* microglia-specific genes and TFs (43).

These findings suggest that cultured microglia are less mature and have a more inflammatory phenotype than *ex vivo* human microglia. As a consequence, transcriptomic discrepancies between *ex vivo* and *in vitro* microglia are challenging the validity of current *in vitro* microglia culture systems to study murine and human microglia physiology and pathology.

Interestingly, Spaethling and coworkers were able to distinguish different brain cell types (neurons, oligodendrocytes, astrocytes, and microglia) after surgically obtained brain tissue was kept in culture for 3 weeks, with k-means clustering of cell-specific markers based on single-cell RNA sequencing data. Although the comparison between cultured microglia with *ex vivo* microglia has not been performed, the current results suggest that *in vitro* microglia lose many genes belonging to the homeostatic gene signature, though microglia can still be segregated from other brain cells in single-cell sequencing analysis (66).

Various studies have aimed to reveal crucial microenvironmental factors in *in vitro* culturing systems, that drive microglial identity toward a more *in vivo*-like state (30, 63). Whereas the astrocyte-secreted factors Tgf-β2, Csf1, and cholesterol are identified as microglial survival factors *in vitro* (63), Tgf-β supplementation of culturing media only led to partial (2, 30, 43) or no (63) re-establishment of an *ex vivo*-like microglial gene expression profile in cultured microglia.

Concluding, the gap in conformity between gene expression profiles of *ex vivo* and *in vitro* microglia is likely caused by the lack of yet unknown CNS microenvironmental factors that keep microglia in a homeostatic state in currently used *in vitro* culture systems.

Microglia Isolation Procedures Affect Their Transcriptome

Next to the *in vitro* cell culture conditions, it should be of note that other *in vitro* procedures, such as the isolation procedure of microglia (tissue dissociation and cell sorting) can affect their gene expression profile, leading to the possibility that currently used reference *ex vivo* microglia expression data might not completely reflect *in vivo* microglia. In fact, it was shown that microglia inflammatory activation markers (*Il1b*, *Tnf*, and *Ccl2*) are already upregulated prior to placing murine microglia into culture, indicating that the isolation procedure by itself already affects the microglial phenotype (63). In addition, evidence indicates that enzymatic dissociation of brain tissue at 37°C leads to the upregulation of inflammatory genes in microglia when compared to mechanical dissociation at 4°C [(29); Eggen et al., unpublished data] or to the cTag-PAPERCLIP method (based on genetically modified animals and does not require tissue dissociation) (67).

In order to minimize the introduction of *in vitro*-induced artifacts when working with microglia in culture, the development of culture conditions and dissociation procedures that result in *in vitro* microglia that highly resemble microglia *in vivo* is of great importance.

MICROGLIAL ACTIVATION STATES FROM A TRANSCRIPTOME POINT OF VIEW

Functional and Morphological Aspects of Microglial Activation

"Microglial activation" is an umbrella term commonly used to describe a great variety of functional and morphological responses of microglia toward different triggers including stress (= homeostatic imbalance), inflammation, or chronic neurodegenerative conditions.

While this term implies that microglia are in a dormant state under healthy/homeostatic conditions, already more than 10 years ago, two photon-imaging of the mouse cortex *in vivo* showed that microglial protrusions are highly motile in order to scan their microenvironment for harmful exogenous and endogenous danger signals (68, 69). In addition, microglia are highly motile under healthy conditions during development. Synaptic pruning, the elimination of excessive, non-active neurons formed early in development, is realized by complement-dependent phagocytic activity of microglia (20, 21).

Microglia in a healthy brain are characterized by a small soma from which ramified protrusions are extending a morphology

evolutionary conserved in different species (70). Classically, microglial activation was associated with an amoeboid-like morphology that enables microglia motility and phagocytic function (22). However, morphological transformation of microglia upon a shift in activation state does not seem to be uniform. Microglial morphologies range from amoeboid-like under inflammatory conditions (71) to hyper-ramification in response to stress (72) and accelerated aging (73), with many intermediate morphologies in between. In addition, different microglia morphologies can also be present at a defined condition such as stroke (74). Thus, it seems that there is, based on morphology, a yet unclear number of microglial activation states and single-cell resolution experiments are required to address that issue in detail. Since the discovery of microglia a century ago, we are aware of the wide range of morphologies microglia can adopt (12), though for most conditions direct links between a specific morphology and functionality of microglia are still unknown.

A Brief History of Categorizing Concepts for Macrophage and Microglial Activation

An early concept that was first postulated for peripheral macrophages is the dichotomous categorization of macrophage activation states into classical activation (M1) or alternative activation (M2), analogous to the Th1 and Th2 nomenclature of T-lymphocytes (75). In an attempt to structure the complexity of microglial activation, the same M1-M2 classification was applied to activation states of microglia. The M1 phenotype is characterized by the production of pro-inflammatory cytokines (*Tnf-α*, *Il-6*, and *Il-1β*), chemokines, and reactive oxygen species leading to an acute immune response. The M2 phenotype is characterized by the production of anti-inflammatory cytokines (*Il-4* and *Il-13*) and facilitates debris clearance, wound healing, and restoration of brain tissue homeostasis (76, 77). It was assumed that microglia react to a stimulus with an M1 phenotype to address pathology and damage, followed by transition to an M2 phenotype in order to execute tissue repair (78). More detailed understanding led to the acceptance that microglial activation states are diverse and that intermediates between M1 and M2 phenotype exist (76). Further development of this concept in the macrophage field suggested to refine the M1-M2 nomenclature by adding the triggering stimulus as an abbreviation to the M1 or M2 classification (77).

Transcriptome studies revisited this concept by disproving the existence of the mutual exclusive M1-M2 polarization states. M1-M2-associated genes (77) were co-expressed by murine monocyte-derived brain macrophages/microglia in the context of traumatic brain injury (79) and ALS (31). Moreover, transcriptome-based network analysis of human monocyte-derived macrophages exposed to 29 different stimuli *in vitro* revealed that each stimulus triggered the expression of a distinct transcription profile. These profiles expand far beyond the M1-M2-associated transcription profiles and under some conditions, M1- and M2-markers are solely expressed at baseline level (80). This study indicates that the concept of an activation spectrum in between the M1-M2 extremes is inadequate.

Recent studies have thus led to the abandonment of this static and outdated M1-M2 concept of microglial (81) and macrophage activation (82) states and point toward the adaption of a so-called "multidimensional concept." This concept incorporates ontogeny, microenvironmental signals, as well as present and past endogenous and exogenous stress signals (83). Such a concept would be in line with current knowledge gained from (single cell) transcriptome and epigenome studies about the great variety of microglial activation states specific to different conditions including aging and neurodegenerative diseases (84).

The Microglial Transcriptome During Aging

Murine Aged Microglia

During aging, microglia undergo several phenotypic changes including in morphology and function (85). The microglial phenotype in aging was extensively studied in a mouse model of accelerated aging that is marked by genotoxic stress due to deficiency of the DNA-repair protein Ercc1. Microglia in generic *Ercc1* mutant mice have a hyper-ramified morphology accompanied by increased proliferation rates. Upon LPS stimulation, microglia from *Ercc1* mutant mice show enhanced expression of pro-inflammatory cytokines (*Il-1β*, *Il-6*, and *Tnf-α*), enhanced phagocytic activity, and reactive oxygen species production when compared to wild-type mice. This exaggerated responsiveness of microglia in aged and in *Ercc1* deficient, accelerated-aging mice is referred to as priming. The primed immune state was confirmed by transcriptional profile analysis, identifying an upregulation of genes associated with immune-related signaling pathways (73). This microglial phenotype was also observed in mice where the *Ercc1* deficiency was targeted to forebrain neurons. These data suggest that genotoxic stress in neurons could induce the observed primed state in microglia.

Overall, aging seems to induce a phagocytic and antigen-presentation gene expression profile when microglial transcriptomes of young and old mice are compared. Microarray analysis of pure microglia from young and old mice showed that aged microglia obtain a gene expression profile that is characterized by upregulation of genes involved in phagocytosis (including *Clec7a* and *Axl*), antigen processing and antigen presentation, interferon and cytokine signaling, as well as lipid homeostasis (including *Apoe*). The increased phagocytic activity in aged/senescent microglia is confirmed by a functional phagocytosis assay. Primed microglia are primarily detected in the white matter of the aging murine brain (86).

Similarly Orre and coworkers (87), identified 482 genes (e.g., *Slp1*, *Apoe*, *Il1r2*, and *Ccr6*) more abundantly expressed in cortical microglia at least by twofold in aged mice (15–18 months) when compared to younger mice (2.5 months). These genes are involved in processes such as vesicle release, zinc ion binding, positive regulation of cell proliferation, lymphocyte activation, and inflammatory response, indicating increased microglia-neuron signaling and an inflammatory status within the aging murine brain (87).

Interestingly, microglia in different regions of the mouse brain show divergent sensitivities to aging. Mainly genes involved in immune regulatory processes are differentially expressed in microglia upon aging. Cerebellar microglia seem to be most prone to aging-induced transcriptional differences, as they

differentially express more than the double number of genes at 22 months of age, when compared to cortical, hippocampal, and striatal microglia of that age. Most of the differentially expressed genes in 22 months old microglia were upregulated genes involved in immunoregulatory functions. Age-related transcriptomic changes in cortical and cerebellar microglia occur relatively consistent during early (4–12 months) and late (12–22 months) aging. Gene expression changes during early aging (4–12 months) are most prominent in the striatum and during late aging (12–22 months) in the hippocampus. Microglia lose the expression of homeostatic microglial signature genes such as *P2ry12/13*, *Tmem119*, and *Fcrls*, most prominently in the cerebellum and to a lesser extent in the hippocampus, cortex, and striatum. These findings suggest that in addition to age-induced effects on microglia in the white matter, age-associated changes in microglia occur in a brain region-specific manner (40, 86).

In contrast to the general notion that microglia obtain a primed profile (40, 86, 87) and are neurotoxic during aging and age-related diseases (88), Hickman and coworkers identified a neuroprotective gene expression profile of microglia derived from the entire brain in aged mice (24 months) due to upregulation of genes involved among others in the Stat3 and Neuregulin-1 pathways. Aging affects the microglia sensome: receptors for endogenous ligands are downregulated while receptors for microbial ligands are upregulated (33).

Human Aged Microglia

An age-related increase in immunoreactivity for inflammatory-related microglial markers, CD68 and HLA-DR, as well as increased binding of a PET tracer for activated microglia ([^{11}C]-(R)-PK11195) has been identified in the white matter of human postmortem brain tissue (86). Whereas these findings indicate that similar to mouse, human microglia adopt a more activated phenotype during aging, transcriptomic analysis identified that the overlap in genes that change expression during aging in mouse and human is very limited. Of note, mouse and human microglia overlap extensively with respect to the expression of homeostatic signature genes (39).

In the human transcriptomic dataset of Galatro and coworkers (39), 572 genes are differentially expressed in relation to the age of the donor. 212 genes are increased and 360 genes decreased in expression, and many of these genes are related to cytoskeleton, motility, and immune response processes. The top 100 most differential expressed genes in human microglia during aging are associated with actin (dis)assembly, cell surface receptors, and genes involved in cell adhesion and axonal guidance. Upregulated genes are mainly associated with actin (dis)assembly and motility, indicating a loss of microglia motility and migration in aged human microglia, a factor that might contribute to age-related CNS diseases. Genes involved in cell adhesion and axonal guidance and the sensome cell surface receptors are partially upregulated and downregulated (e.g., *P2RY12*) (39).

The overlap in genes that are differentially expressed during aging between humans and mice is very limited (39). Only 14 upregulated genes overlapped between the human and mouse data and are involved in positive regulation of cell-matrix adhesion. Nine genes have a reduced expression during aging in both human and mice, identifying genes as *ETS1*, *SEMA7A*, *MRC2*, *PSTPIP1*, and *EMP2* (39). Concluding, the response of microglia to aging is different in mouse and human.

Although not yet completely understood, microglia seem to obtain an age-induced immune-activated phenotype during aging, which likely contributes to the pathology of neurodegenerative diseases including AD and PD (85, 88–90). In contrast to mice, human microglia also adapted their cytoskeleton signaling during aging (7).

The Transcriptomic Point of View on Activated Microglial Phenotypes in Neurodegenerative Diseases

A shared feature among different neurodegenerative disorders is microglia-mediated neuroinflammation (91). This type of microglial activation is a first line of defense in the CNS, but is also described as harmful (91, 92). Microglial activation can be observed in different neurodegenerative diseases in which microglia obtain specific phenotypes.

Alzheimer's Disease

Several AD studies reported activated microglia surrounding Aβ plaques (34, 35, 93, 94). Plaque-associated microglia in the 5XFAD AD-mouse model (co-expresses five mutations associated with familial AD) contain upregulated sets of genes that overlap with the primed microglia transcriptional profile (95), that is characterized by enrichment of genes involved in among others immune and phagocytic processes, like *Apoe*, *Axl*, and *Clec7a*. Key protein regulators of those upregulated genes are *Tyrobp* (*Dap12*) and *CD11c* (*Itgax*). Of note, plaque-associated microglia in 5XFAD mice show an upregulation of phagocytosis-associated genes. Interestingly, the same phagocytic markers, *APOE*, *AXL*, *TREM2*, and *HLA-DR* are shown to be higher expressed in microglia surrounding dense-core plaques of early onset AD human postmortem tissue, when compared to late onset AD (LOAD) (94). In contrast to the finding that the expression of *TYROBP* is unaltered between plaque and non-plaque-associated microglia of LOAD postmortem brain tissue (94), *TYROBP* is identified as a key regulator of microglial-associated genes, based on the construction of a molecular network from autopsied whole brain samples of 1647 LOAD and non-demented subjects (96). Kamphuis and coworkers identified two distinct subsets (CD11c$^-$ and CD11c$^+$) in the CD11b$^+$ microglia population surrounding Aβ plaques in APP/PS1 mice. Transcriptional alterations are more abundant in the CD11c$^+$ population when compared to CD11c$^-$ microglia, including an upregulation of *Clec7a, Itgam*, *Ctsb*, and *Cst7* expression. The CD11c$^+$ microglial population is enriched for genes involved in a dampened immune response, carbohydrate and lipid metabolism, phagocytosis and lysosomal degradation, suggesting that the CD11c$^+$ population is active in the clearance of amyloid deposition by possibly increased phagocytic and lysosomal activity and restriction of the inflammatory response (93). By contrast, it was recently observed that innate immune activity (inflammasome activity) of microglia leads to Aβ accumulation in APP/PS1 mice. Although not distinguishing between CD11c microglia subsets, it is shown that

microglia secrete inflammasome-associated adaptor proteins, called apoptosis-associated speck-like proteins containing a CARD (caspase recruitment domain; ASC). ASC proteins can go through a cascade of modifications that lead to the assembly of large extracellular paranuclear ASC protein complexes, called ASC specks. These ASC specks are prone to bind Aβ deposits throughout brain tissue of AD patients and APP/PS1 transgenic mice. They are identified as the key contributors to several AD characteristics, such as the formation of plaques and spatial memory loss (97).

Concluding, the contradiction between the hypothesized function of $CD11c^+$ microglia (clearance of Aβ plaques) and the proven function of $CD11b^+$ microglia (augmentation of Aβ plaques), might be explained by the fact that $CD11c^+$ microglia only constitute approximately 23% of the total activated CD11b microglial population (93), whereby its potential neuroprotective function might be overruled by the neurotoxic function of the remaining microglia.

Interestingly, single-cell analysis of hippocampal microglia from CK-p25 mice, a mouse model of severe AD-like neurodegeneration, identified a stepwise microglial gene expression trajectory in response to neurodegeneration. One week after CK-p25 induction, microglia possess an early-response state, which is hallmarked by an upregulation of genes involved in cell cycle, DNA replication, and repair. Increased incorporation of the thymidine analog EdU (5-Ethynyl-2′-deoxyuridine) and microglial density in CK-p25 mice 1 week after induction confirmed microglial proliferation in response to early neurodegeneration. Two and six weeks after disease induction, late-response microglia, show upregulation of immune response-related genes, such as *Ccl3/4*, *Apoe*, *Axl*, and *H2D1*. This microglial phenotype can be divided into two immune-activated subtypes that are marked by co-regulated genes induced by interferon type I (antiviral and interferon response genes) and II (MHC-II complex-related genes), respectively. Whether these microglial phenotypes have neuroprotective or neurotoxic functions remains unknown (98).

Multiple Sclerosis

Multiple sclerosis lesions have been categorized into (1) preactive lesions, characterized by microglia activation in the absence of overt demyelination, (2) active lesions with massive infiltration of microglia and monocyte-derived macrophages, (3) mixed active/inactive lesions that consists of a hypocellular center and a foamy macrophage/microglia-enriched rim with partial demyelination, and (4) inactive lesions that are absent of cells and completely demyelinated. MS lesions are surrounded by normal appearing white matter, where microglial activation may occur as well (99). It is very difficult to interpret the distinct roles of microglia and monocyte-derived macrophages in MS pathology, since both macrophage populations are present in MS lesions (6). This mixed cell population of microglia and monocyte-derived macrophages has been investigated on transcriptomic level in human MS tissue (38, 100). Attempts have been made to decipher the role of microglia and peripheral monocytes in an experimental autoimmune encephalomyelitis (EAE, myelin-oligodendrocyte-glycoprotein peptide (MOG)-induced) mouse model for MS (37, 101). Yamasaki and coworkers distinguished microglia from monocyte-derived macrophages by the use of genetically modified mice that express fluorescent proteins (green or red fluorescent proteins) expressed under the control of a microglial (Cx3cr1) or monocytic (Ccr2) promoter. The study showed that microglia and monocyte-derived macrophages from the same tissue have different phenotypes in EAE. At the onset and peak of disease, microglia upregulated genes that are involved in the complement system (e.g., *C1qa*, *C3*, and *C4*), chemotaxis (e.g., *Ccl2/4*), cell migration, and acute inflammation (e.g., *Il1β* and *Tnf*) and downregulated genes that are involved in cell metabolism. By contrast, the gene expression profile of monocyte-derived macrophages is characterized by phagocytosis-, autophagy-, and cell clearance-related genes. Along with the finding that solely monocyte-derived macrophages form contacts at nodes of Ranvier, it was suggested that monocyte-derived macrophages initiate demyelination at EAE onset, whereas microglia seem to be responsible for the attraction of monocyte-derived macrophages to the CNS and to clear debris (37). Similar results have been reported in a study that used CD44 protein expression levels to distinguish microglia ($CD44^{low}$) from monocyte-derived macrophages ($CD44^{high}$) in EAE. RNA expression analysis of microglia and monocyte-derived macrophages from EAE mouse brain tissue reveal that macrophages display a more pronounced immune activation phenotype at the peak of EAE, characterized by common activation markers such as *MHCII*, *CD40*, and *CD86*. In comparison with monocyte-derived macrophages, microglia upregulate genes involved in uptake of apoptotic cells, the complement signaling, and chemotaxis at the peak of EAE (101).

In conclusion, these above studies suggest that monocyte-derived macrophages and microglia have different roles during the disease progression of EAE. Monocyte-derived macrophages seem to be the mediators of demyelination, whereas microglia are primarily responsible for the induction of peripheral infiltration to the CNS and clearance of apoptotic neurons in EAE. Whether these different macrophage phenotypes exist in human MS pathology as well needs to be addressed in future experiments.

Parkinson's Disease

Microglial activation is initiated by several components, whereas one of the most frequently altered genes in familial PD is α-synuclein (91). Overexpression of a-synuclein led to the activation of BV2 cells, a microglial cell line, measured by an increase in cytokine production (102). Since *in vitro* cultured microglia do not resemble *in vivo* microglia (see The Transcriptome of *In Vitro* and *Ex Vivo* Microglia Is Different), the microglial phenotype associated with PD *in vivo* yet remains to be elucidated.

Whole tissue lysate small RNA sequence analysis of postmortem prefrontal cortex of PD patients (demented and non-demented) and control subjects, identified a set of 29 PD-related miRNAs (103). Interestingly two *Pu.1* related micro RNA's (miR146a and miR-155) (36, 104) are upregulated in PD subjects, suggesting that microglia might be activated in human PD. Single-cell laser captured microglia of human postmortem PD brain tissue were used to identify microglial gene expression

in PD (45). Overall, the most differentially expressed genes in microglia derived from PD subjects compared to control subjects are involved in functions such as aldosterone synthesis and secretion, positive regulation of protein complex assembly, focal adhesion assembly, tonic smooth muscle contraction, and positive regulation of reactive oxygen species biosynthetic processes. 313 genes are differently expressed in microglia located in the substantia nigra when compared to the CA1 hippocampal region of PD patients. These genes are involved in the behavior, regulation of transport, and synaptic transmission processes. The above findings indicate regional differences in microglial functioning in PD (45). Concluding, overall the expression pattern of genes in PD points toward microglial activation. Unfortunately, the small number of PD microglia transcript studies limit the current conclusion.

Amyotrophic Lateral Sclerosis

An activated microglial phenotype was reported in the transgenic SOD1-G93A mouse model for ALS, that contains mutations in the human superoxide dismutase 1 gene. The microglial phenotype is identified as a neurodegeneration-specific phenotype and differs from LPS-activated microglia as well as from M1- or M2 macrophages (31). It was shown that microglia in SOD1-G93A mice simultaneously upregulate neurotoxic and neuroprotective factors as well as pro-inflammatory-related genes (e.g., *Tnfα* and *Il-1β*). In addition, an upregulation of genes that have been associated with AD, including *Trem2*, *Tyrobp*, and *Apoe* associated with AD are found in microglia from SOD1-G93A mice. Furthermore, *Apoe* is also upregulated in both SOD1-G93A mice and ALS subjects (sporadic and familial) (36). At least for *in vitro* conditions, *Apoe* seems to play a role in forcing the "surveilling" microglia toward an immune-activated (M1-like) phenotype (36). In addition, a downregulation of the homeostatic microglial signature genes (including *P2ry12* and *CD39*), transcriptional factors (including *Egr1*, *Atf3*, *Fos*, and *Mafb*), developmental genes (such as *Tgfb1*, *Tgfbr1*, and *Csf1r*), and genes related to phagocytic ability and cell migration was described in SOD1-G93A mice (36). This indicates a suppression of several homeostatic microglial functions. Interestingly, the microRNA-155 (miR-155) is identified to be upregulated in microglia of SOD1-G93A mice as well as in spinal cord tissue of ALS subjects (36).

Genetic ablation of miR-155 in SOD1 mice causes a delay of the disease onset, an extend of the animal survival rates, and reversed the expression of SOD1-related upregulation of inflammatory genes and downregulation of homeostatic genes in microglia. In conclusion, miR-155 seems to be an important factor in driving the phenotypic switch from homeostatic to SOD1-specific activation microglia. Therefore, miR-155 might be a potential new therapeutic target in ALS (36).

MICROGLIAL CORE GENE SIGNATURES ASSOCIATED WITH DIFFERENT DISEASES

Next to the identification of disease-specific microglial transcriptomes, in the past years several studies have addressed a core profile of microglial genes that are dysregulated in multiple neurodegenerative diseases (34, 35, 95).

Massively parallel single-cell RNA-sequencing of $CD45^+$ immune cells revealed among others the presence of three novel microglial transcriptional subpopulations in 5XFAD mice that are not present in wild-type animals. Two of them are characterized by the expression of genes involved in lipid metabolism and phagocytosis and are specifically located near Aβ plaques in the cortex of AD mice, called disease-associated microglia (DAM). DAMs seem to undergo a cascade of subsequent changes in gene expression profiles alongside the progression of the disease. The first step includes an increased expression of *Tyrobp*, *Apoe*, and *B2m* and reduced expression of microglial homeostatic signature genes (*Cx3cr1* and *P2ry12*). There seems to be a *Trem2*-dependency from the second step onward together with the upregulation of genes involved in lipid metabolism and phagocytic activity, such as *Lpl*, *Cst7*, *Axl*, and *Clec7a*. DAMs have also been identified in postmortem human AD tissue and in a mouse model for ALS (mSOD1 mice). Moreover, appearance of DAMs was observed when $CD11b^+$ immune cells are compared between brains of young (7 weeks old) and aged (20 months old) mice. These findings suggest that DAMs (35) might have a general neuroprotective function involved in the clearance of accumulating proteins observed in aged and age-related neurodegenerative diseases brain tissue (105).

In contrast to the hypothesized neuroprotective function of DAMs, investigation of bulk microglial transcriptomes in different disease models led to the identification of a microglial neurodegenerative/-toxic (MGnD) phenotype that is dependent on the Trem2-Apoe pathway. These MGnD are found adjacent to Aβ plaques in APP/PS1 mice and human AD postmortem brain tissue and in SOD1, EAE, and aged (17 months) mice. Two major transcriptional changes are observed in MGnD: (1) the downregulation of microglial homeostatic genes [including *Tgfb(r)*, *Hexb*, *P2ry12*, and *Cx3cr1*] and TFs (including *Mef2a*, *Mafb*, and *Sall1*) and (2) the upregulation of inflammatory genes (including *Axl*, *Itgax*, *Clec7a*, and *Apoe*), leading to a switch from a homeostatic to a neurodegenerative/-toxic microglial phenotype. This switch seems to be induced by the phagocytosis of apoptotic neurons and is dependent on Trem2-Apoe signaling and accompanied by a suppression of the Tgfβ pathway. Depletion of *Trem2* in APP/PS1 and SOD1 mice suppresses the expression of inflammatory genes, including *Apoe*, restores the homeostatic microglia gene signature and functions, and also alleviates disease-specific characterizations such as reduced Aβ plaques in APP/PS1 mice and reduced expression of miR-155 in SOD1 mice. Interestingly, the microglial homeostatic phenotype seems to be preserved in human AD patients who carry a mutation in the TREM2 gene when compared to AD patients with wild-type TREM2 (34).

Compared to the results of Krasemann and Keren-Shaul, a similar set of genes, including *Apoe*, *Axl*, *Itgax*, *Lgals3*, *Clec7a*, *MHC-II*, and *Cxcr4*, was identified as a commonly upregulated network of genes in different mouse models of aging [physiological aging and accelerated aging (24 months; Ercc1 deficient)] and murine disease models (APP/PS1 and SOD1) when compared to acute immune activation with LPS. This network is classified

as the "microglia priming" network and is associated with functions involved in AD signaling, antigen presentation, lysosome and phagosome pathway. In addition, it was found that microglial homeostatic genes are suppressed in this "primed" network. The "primed" gene expression network related to microglial activation is contrasting with an "acute" activation network, specific for acute microglial inflammatory response to LPS that is marked by an upregulation of genes involved in ribosome, TLR signaling, and NOD-like receptor signaling (95).

Summarizing, different studies identified a microglial gene signature associated with multiple diseases that is marked by the downregulation of microglial homeostatic signature genes and the upregulation of genes associated with inflammation, phagocytosis, and lipid metabolism, whereby the two genes *Apoe* and *Trem2* seemed to be crucial players. Whereas the upregulation of phagocytic genes might imply neuroprotective functions of microglia (35, 93), recent studies show that microglia also seem to have a neurodegenerative/-toxic function in multiple neurodegenerative diseases (34, 97).

By comparing these aging- and neurodegeneration-associated microglial core gene signatures (34, 35, 95), an overlap between the different gene signatures (modules) is determined and visualized in **Figure 1**. A summary of the overlapping aging- and neurodegeneration-associated genes is depicted in the Venn diagram and are is listed in **Table 1** where the annotation of gene functions is done with stringDB (106). There are three genes identified that are shared among these three datasets: *APOE*, *AXL*, and *IGF1*, identifying a very limited overlap of microglial-associated disease genes between three studies that have investigated similar age-related disease mouse models.

DISCUSSION

The diversity of microglial phenotypes can be metaphorically imagined as the manifold compositions of colorful crystals seen in a kaleidoscope, whereby a change in (micro)environment is functionally equivalent to a rotation of the kaleidoscope. Technological developments in next-generation sequencing provide the possibility to reveal cell-type specific transcriptomes at single-cell resolution. Over the last 5 years, different studies have concordantly established the microglial gene signatures in mice and humans under homeostatic conditions (2, 3, 29–33, 39, 43). In addition, microglia-specific gene signatures in different neurological disease contexts were investigated. Summarizing, it was shown that the expression of many of the homeostatic microglial signature genes are repressed during aging and age-related CNS diseases. It is expected that more transcriptomic studies will follow, especially in regard to neuropsychiatric disease, from which transcriptome datasets are currently very sparse.

Identification of microglial genes that are highly expressed and shared between different neurodegenerative diseases and aging might result in the identification of regulators that can alter affected pathways in multiple diseases. Thus far, three core signatures of microglia associated with multiple diseases have been identified (34, 35, 95). Although, microglial transcriptomes under similar conditions (aging and models for AD, MS, and ALS) have been evaluated in these three studies, only a limited overlap of genes is identified (see **Figure 1**), indicating that other factors might have influenced the transcriptional outcome. **Figure 2** depicts examples of parameters, such as development, aging, sex, diseases, and experimental procedures, that were shown to influence microglial transcriptomes and might have led to the different results of the above-mentioned studies. In order to identify a "pure" microglial core signature related to multiple CNS diseases, the only deviating factor between the compared datasets should be "disease/disease model." Obviously, this criterion is difficult to meet in a laboratory setup and is mere impossible in datasets derived from the human population. Conversely, the question arises whether the overlap of disease-associated microglial datasets, that are influenced by other factors, indeed identifies a universal regulator that is most important for all different investigated disease phenotypes. In view of the fact that

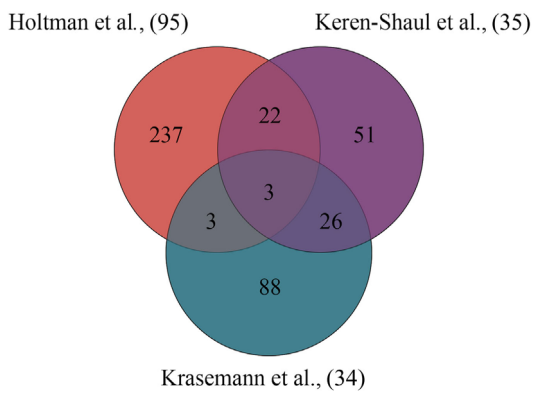

FIGURE 1 | Overlapping gene signatures of microglial core profiles associated with multiple diseases identified in three independent studies. Using the "primed" module by Holtman and coworkers (95), the gene profile of disease-associated microglia identified by Keren-Shaul and coworkers (35) and the gene profile of the microglial neurodegenerative phenotype identified by Krasemann and coworkers (34), three genes were identified to be shared among the three microglia datasets associated with multiple diseases.

TABLE 1 | Gene overlap of different microglial core profiles associated with multiple diseases.

Compared studies	Overlapping genes
Keren-Shaul et al. (35) and Krasemann et al. (34)	*BIN1*, *CCR5*, *CD34*, *CKB*, *CTSD*, *CX3CR1*, *ENTPD1*, *EPB4.1L2*, *F11R*, *FSCN1*, *GPR34*, *GPR56*, *LGMN*, *LTC4S*, *OLFML3*, *P2RY12*, *P2RY13*, *PMEPA1*, *RHOB*, *SERPINE2*, *SIGLECH*, *SLCO2B1*, *SPARC*, *SYNGR1*, *TMEM119*, and *TREM2*
Holtman et al. (95) and Keren-Shaul et al. (35)	*ANK*, *APLP2*, *B2M*, *CD52*, *CD68*, *CD9*, *CLEC7A*, *CSF1*, *CST7*, *CTSB*, *CTSZ*, *EEF1B2*, *GRN*, *GUSB*, *H2-K1*, *HIF1A*, *ITGAX*, *LGALS3BP*, *NPC2*, *PLD3*, *PSAT1*, and *TYROBP*
Holtman et al. (95) and Krasemann et al. (34)	*CXCL16*, *CCL5*, and *GAS7*
Holtman et al. (95), Krasemann et al. (34), and Keren-Shaul et al. (35)	*APOE*, *AXL*, and *IGF1*

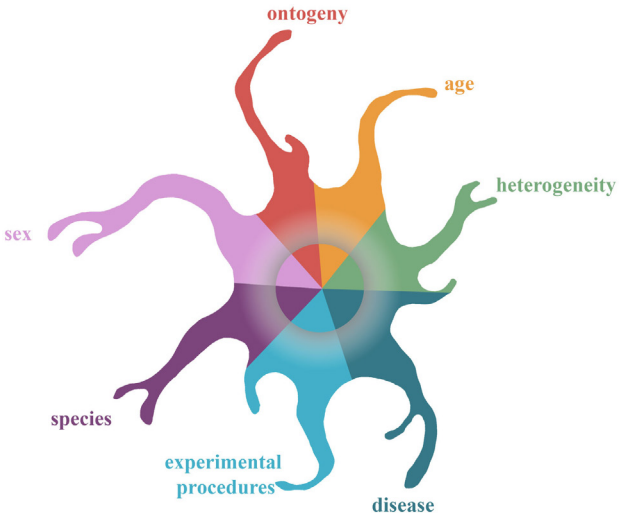

FIGURE 2 | The kaleidoscope of microglial phenotypes: complexity of determining a microglial core profile associated with multiple diseases. This figure depicts examples of factors that impinge on the microglia epigenome and transcriptome. Seven factors are depicted that have been shown to influence the microglial transcriptome: brain region, ontogeny, disease, age, sex, experimental procedures, and species. The influence of these factors on the transcriptome converges in the center of the gray rim. This rim represents the imprint of these factors on the epigenome, which yet remains to be revealed.

research is often pursued in order to identify drug targets for human diseases, it is questionable if identification of a multiple disease-associated core signature of microglia will lead to a universal drug target for multiple diseases or whether disease-specific regulators might be more suitable as drug targets.

Current studies have delineated the influences of several environmental events (see **Figure 2**) on the microglial transcriptome. These findings indicated that microglia "imprint" different events on their (epi)genome and that imprints that occur early in life can possibly influence microglial phenotype over the entire lifespan of an animal. Although the precise microglial turnover rate is not yet completely clarified, microglia seem to be quite long-lived cells further supporting a potential role of epigenetics in regulating microglial function. Investigation of the microglial epigenetic landscape under homeostasis has already provided valuable insight in the (micro)environmental-induced dynamics of chromatin modifications and how these affect gene expression (2, 3, 43). Although currently unknown, investigation of the microglial epigenetic landscape in context of CNS diseases would reveal the link between the CNS-disease related microenvironment and the corresponding transcriptome and would definitely contribute to a better understanding of the role of microglia in the pathophysiology of CNS diseases.

One of the recent developments in sequencing technology is the ability to decipher transcriptomes and epigenomes at the resolution of a single cell (107, 108). The identification of single-cell transcriptomes and epigenomes will greatly facilitate the characterization of distinct microglial phenotypes under particular conditions, ranging from (ab)normal development to neurodegenerative disease, and allow for a detailed and sophisticated functional classification of microglial phenotypes.

AUTHOR CONTRIBUTIONS

MD and LK wrote the manuscript. BE and EB gave feedback and edited the manuscript.

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