Clinical and Developmental Immunology

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Guest Editors: Anirban Ghosh, Wolfgang J. Streit, Luisa Minghetti, and Anirban Basu

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Editorial Board

Contents

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Editorial

Microglia in Development and Disease

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The first special issue on microglia was published in 1993 in *Glia* and others followed in regular, roughly ten-year intervals. Clearly, the importance of microglia has been recognized since the 1980s while the journal *Glia* has (appropriately) taken the lead in regularly reviewing microglial biology. Also there have been others. The current issue is the first one entirely devoted to microglia and perhaps not the last since research interest in this field has grown exponentially in the last two decades and continues to thrive. Why is the microglia field booming with no obvious end in sight? It is because microglia have become recognized as incredibly diverse and complex cellular players not only in virtually all kinds of brain pathology, but in many aspects of normal brain function as well.

There is a growing realization that as the brain's immune system, these little guys might be of critical importance in terms of normal brain function, neurological disease pathogenesis (neural tissue degeneration), and restoring homeostasis after sudden destructive brain injury (neural tissue regeneration). In present years, the cells have been found to have direct and active involvement to prune and shape neuronal circuits, maintaining and monitoring overall neuronal microenvironmental health. Recent indications of their potential importance in neuropsychiatric disorders, cognitive behavior, spatial learning, and even masculinisation are now nudging microglia research into newer arenas. Further advancement in *in vivo* imaging and molecular probing techniques and innovative improvisation of different methodologies has not only opened up diverse aspects of microglia biology but also provided deeper insight into existing knowledge. However, with many advances in understanding of microglia, some basic queries and controversies regarding microglia/brain macrophages, their cellular characteristics and demarcation, origin and colonization in developing, adult and diseased brains, and their double-edged behavior and polarization in different normal and pathogenic situations still remain. This special issue represents an attempt to present a few of the many areas in which advances in microglial research have influenced our understanding of neurosciences. A total of ten articles have been assembled to discuss some of the newer as well as more traditional fields of microglia research. This special issue is a collection of articles with strong emphasis on the molecular interpretation of activities of microglia in normal and diseased brain.

The involvement of microglia in normal and pathological brain function is reflected in their molecular makeup, which presents as an incredibly diverse array of surface membrane receptors capable of receiving and processing myriads of signals created constantly in the CNS microenvironment. How such signals may regulate subsequent gene expression and microglial behavior is discussed here by J. Guedes et al. who take a close look at microglial micro-RNAs, a topic likely to receive additional attention for years to come. Another up-and-coming theme is the potential involvement of microglia in some of the more subtle brain pathologies represented by neuropsychiatric disorders where a clear pathological correlate in terms of neurons is often not apparent, and where the emerging concept of microglial involvement still remains

a bit mysterious. L. R. Frick et al., who address this issue, discuss microglial involvement in neuropsychiatric disorders in the context of microglial pathology, an aspect of microglia biology that has not been addressed much in previous special issues but one that is gaining ground fast and is going to be around. The current issue also contains several contributions on persistent mainstream topics, perhaps most notably and traditionally microglial roles in neuroinflammatory disease, that is, multiple sclerosis and its various animal models, evidenced in excellent contributions by K. S. Rawji and V. W. Yong, H. Koennecke and I. Bechmann, D. Chatterjee et al., and T. Goldmann and M. Prinz, each illuminating slightly different aspects of microglial involvement and participation in demyelination and neurodegeneration. The controversial (i.e., unknown) roles that microglia may play in malignant glioma growth and invasion which continues to fascinate brain tumor biologists and their views are represented in contributions by A. C. C. da Fonseca and B. Badie and by J. Wei et al. Finally, there are an update on microglial immunophenotypes in the context of neuropathic pain by K. Li et al. and a beautiful review on microglial activities in the leech, that well-known invertebrate which can regenerate its CNS from scratch with the help of microglia.

Acknowledgments

It was our great enjoyment to be involved and working together in developing such an issue. We convey our thanks to the contributors and reviewers who shared their expertise and time. We hope that readers will enjoy reading the issue.

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Review Article

The Role of Microglia and Matrix Metalloproteinases Involvement in Neuroinflammation and Gliomas

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Matrix metalloproteinases (MMPs) are involved in the pathogenesis of neuroinflammatory diseases (such as multiple sclerosis) as well as in the expansion of malignant gliomas because they facilitate penetration of anatomical barriers (such as the glia limitans) and migration within the neuropil. This review elucidates pathomechanisms and summarizes the current knowledge of the involvement of MMPs in neuroinflammation and glioma, invasion highlighting microglia as major sources of MMPs. The induction of expression, suppression, and multiple pathways of function of MMPs in these scenarios will also be discussed. Understanding the induction and action of MMPs might provide valuable information and reveal attractive targets for future therapeutic strategies.

1. Barriers from Blood to Brain

Influx of inflammatory cells into the neuropil is a hallmark of neuroinflammation (e.g., in multiple sclerosis (MS) [1], and respective mechanisms have been studied extensively in experimental autoimmune encephalomyelitis (EAE), an animal model for multiple sclerosis. Initially, leukocytes migrate across vascular walls and accumulate in the perivascular space. This perivascular "cuffing" [2], however, is only the first step in neuroinflammation because immune cells need to pass the glia limitans and its basement membrane to reach the parenchyma proper in a second, differentially regulated step [3]. While the endothelium does not provide an insurmountable barrier for activated T and B cells under certain (experimental) conditions [4, 5], the glia limitans and the parenchymal basal lamina represent more strictly regulated, secondary barriers [3]. Importantly, clinical symptoms only occur after the penetration of the parenchymal basal lamina (BM), which is formed by a variety of organized extracellular matrix (ECM) components build by astrocytic endfeet of the glia limitans.

There is strong evidence that inducible proteases, known as matrix metalloproteinases (MMPs), are involved in the second step of neuroinflammation [3, 6–10]. The unique features of different, highly specialized, basal laminae rely on their major constituents: collagen IV and laminin predominant are whereas collagen type V, proteoglycans, and glycoproteins are additional constituents [11]. Collagen types IV and V are, unlike other collagens, structurally organized in a nonfibrillar, multilayer network that is resistant to nonspecific proteolytic degradation. Noteworthy is the existence of different laminin isoforms in the specialized basement membrane (BM) of the vessel and the BM of the glia limitans. While the vascular BM exhibits laminin 8 and laminin 10, the BM of the glia limitans is characterized by laminin 1 and laminin 2 [12]. Dystroglycan is a transmembrane receptor that anchors astrocyte endfeet to the parenchymal BM [13–15] via high-affinity interactions with laminin 1 and 2. Dystroglycan was identified as a specific substrate of MMP-2 and MMP-9 [16]. Thus MMPs, secreted by juxtavascular microglia, might control the ECM composition, and as a consequence MMPs are involved in the integrity and function of the glia limitans.

2. The MMP Family

The MMPs are a family of zinc containing endoproteinases that share structural domains but differ in substrate specificity, cellular sources, and inducibility. The major function

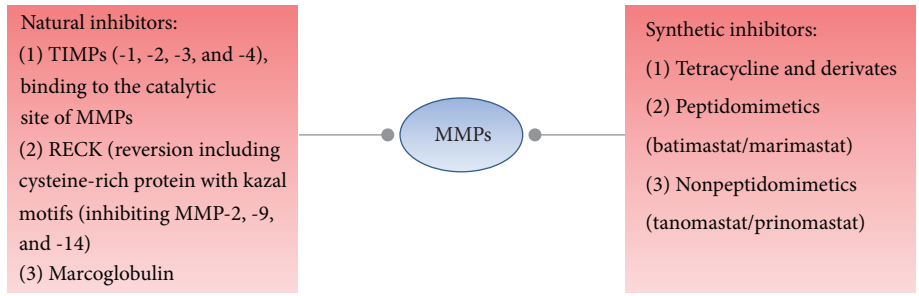

Figure 1: MMP inhibition is possible by targeted natural as well as synthetic inhibitors. References: Visse and Nagase [18]; Oh et al. [201]; Coussens et al. [202]; Overall and López-Otín [203].

is the degradation and remodeling of all components of the ECM. As a group of more than 20 structurally related enzymes, they can be divided according to their substrate affinity profile: gelatinases (MMP-2 and -9), interstitial collagenases (MMP-1, -8, and -13), broad-specific stromelysins (MMP-7 and -13), and other variants (see Table 1) [17]. Together, the MMP substrate repertoire includes the extracellular matrix components, fibrillar collagens, elastin as well as matrix proteoglycan core proteins, and furthermore an expanding range of nonmatrix substrates [18, 19]. MMPs are synthesized in an inactive proform that is activated extracellularly by proteolytic cleavage under the regulation of several inflammatory mediators, including cytokines, chemokines, free radicals and steroids [20, 21]. Moreover certain MMPs are able to activate others; for example, MMP-12 was shown to activate MMP-2 and MMP-3, thereby leading to an exacerbation of proteolytic processes [22].

As proteolytic enzymes, MMPs have important roles in development and physiology. They are thus linked to physiological activities in the CNS, such as myelin formation, axonal growth, angiogenesis, and regeneration [23, 24]. In general, a deviant expression or overproduction of these MMPs leads to tissue destruction, and may contribute to brain pathologies such as Alzheimer's disease, ischemia, malignant glioma, and Parkinson's disease [25–29], when not counterbalanced by their physiological inhibitors, the tissue inhibitors of MMPs, TIMPs [18] (see Figure 1). Usually MMPs are under strict control at various levels: gene transcription, synthesis, secretion, activation, inhibition and glycosylation. Therefore, normal adult CNS contains low levels of most MMP members [30], in contrast to various neurological disorders of the CNS in which several MMPs are significantly upregulated [31].

The upregulated MMPs in the CNS have several potentially detrimental roles, including the promotion of neuroinflammation, disruption of the blood brain barrier (BBB) [20, 32], demyelination, and damage to axons and neurons (especially MMP-1 and MMP-2) [33]. MMPs also participate in the inflammatory cascade itself by actions on inflammatory mediators and their receptors [34, 35]. Thereby, several MMPs may act in concert in a so called *MMP cascade* [13]. Moreover, MMPs may contribute indirectly to the expansion of the inflammatory response and tissue damage by generating antigens through the breakdown of myelin or by conversion of inactive membrane bound TNF-α into the active myelinotoxic form [36]. Similar molecules (e.g., TNF receptors, L-selectin, TGF-β and FAS ligand) may, due to the action of MMPs, undergo analogous processes [37]. The definite sources of the activated MMPs are still to be determined: invading T cells may release proinflammatory cytokines that activate glia cells, which are in control of the expression, secretion and balance between MMPs, as well as the secretion of their natural and specific inhibitors (TIMPS).

3. Microglia in Inflammation

The primary immune effector cells of the brain are microglia, which are activated in response to brain injury or inflammatory conditions. Most likely, they play a pivotal role during onset, maintenance, relapse and progression of an inflammatory state. In the course of activation, they do not only release neurotrophic factors (such as nerve growth factor and brain-derived neurotrophic factor), but also neurotoxic factors (e.g., nitric oxide) and proinflammatory cytokines (TNF-α and IL-1) [38, 39]. Thus microglial activation is necessary for host defense, but this comes at the prize of additional "bystander damage" [40]. There is evidence that microglia play a detrimental role in various neurodegenerative diseases [41, 42]. However, ample data demonstrate beneficial roles for microglia, for example, by stimulating myelin repair, removal of toxic proteins from the CNS, and the prevention of chronic neurodegeneration [43, 44]. Microglial activation can be caused by neuronal cell death leading to secretion of signaling molecules (including α-synuclein, neuromelanin, and active forms of MMP-3) [38, 39, 45, 46]. The expression of MMPs, produced in microglia at sites of inflammation upon activation (such as LPS and Con A [47, 48], could be shown in various studies [1, 49, 50]. Particularly the secreted MMP-2 and MMP-9 [16, 51] seem to be the key modulators (Figure 2).

4. MMP-2 and MMP-9 in Inflammation

MMP-2 and MMP-9 are structurally related and share the common feature of a zinc-binding domain. MMP-2 (gelatinase A a 72 kDa type IV collagenase) and MMP-9 (gelatinase B a 92 kDa type IV and type V collagenase) degrade collagens IV and V in their native forms [52]. Besides collagen, MMP-9 targets a variety of other substrates, for example, substance

Table 1: MMP overview. It was found that MMP-4, -5, and -6 were identical with MMP-2 or -3. Data compiled from Parks et al. 2004 [17] and Sbardella et al. 2012 [160].

MMP group	MMP subgroup	Designation	Alternative name	Matrix substrate	Bioactive substrate
Simple hemopexin-containing MMPs	Collagenases	MMP-1	Collagenase-1/ColA/ColB/Fibroblast collagenase/interstitial collagenase	Unclear: Type I and II fibrillar collagens; collagens I, II, III, VII,VIII, X; gelatin; aggrecan; link protein; entactin; tenascin; perlecan	a2-M; a-PI; a1-antichymotrypsin; IGFBP-2, 3, 5; proIL-1b; CTGF
		MMP-8	Neutrophil collagenase	Collagens I, II, III, V, VII, VIII, X; Fn; entactin; tenascin; gelatin; aggrecan; link protein, Mouse CXCL5	a-PI
		MMP-13	Collagenase-3	Collagens I, II, III, IV, VII, IX, X, XIV; aggrecan; gelatin; Fn; tenascin; osteonectin; Ln; perlecan	CTGF; ProTGF-b; MCP-3; a1-antichymotrypsin; plasminogen
	Stromelysins	MMP-3	Stromelysin-1 (transin-1)	E-cadherin, Laminin, type IV collagen, Latent TGF-β1; Aggrecan; decorin; gelatin; Fn; Ln; collagens III, IV, V, IX, X, XI; tenascin; link protein; perlecan; osteonectin; entactin	IGFBP-3; proIL-1b; HB-EGF; CTGF; Ecadherin; a1-antichymotrypsin; a1-PI; a2-M; plasminogen; uPA; pro-MMP-1, 7, 8, 9, 13
		MMP-10	Stromelysin-2	Aggrecan; Ln; Fn; gelatin; collagens III, IV, V, IX, X, XI; tenascin; link protein;	Pro-1, 8, 10
	Others	MMP-12	Macrophage metalloelastase	Latent TNF, Elastin; aggrecan; Fn; collagen IV; gelatin; vitronectin; entactin; osteonectin; Ln; nidogen	Plasminogen; apolipoprotein(a)
		MMP-19	RASI-1	Collagen IV; gelatin; Fn; tenascin; aggrecan; entactin; COMP; Ln; nidogen	IGFBP-3; proIL-1b; HB-EGF; CTGF; E-cadherin; a1-antichymotrypsin; a1-PI; a2-M; plasminogen; uPA; pro-MMP-1, 7, 8, 9, 13
		MMP-20	Enamelysin	Amelogenin; aggrecan; gelatin; COMP	Unknown
		MMP-27	None	Unknown	Unknown
Gelatin-binding MMPs	Gelatinases	MMP-2	Gelatinase A/72kDa gelatinase	CCL-7/CXCL12, gelatin; collagens I, IV, V, VII, X, XI, XIV; Ln; Fn; elastin;aggrecan; osteonectin; link protein	ProTGF-b; FGF receptor I; MCP-3; IGFBP-5; proIL-1b; galectin-3; plasminogen;
		MMP-9	Gelatinase-B	Zona occludens1, α1-Antiproteinase, latent TGF-β1, latent VEGF, Fibrin, NG2 proteoglycan; gelatin; collagens I, III, IV, V, VII, X, XII; elastin; entactin; aggrecan; Fn; link protein; vitronectin; N-telopeptide of collagen I	ProTGF-b; IL-2 receptor a; Kit-L; IGFBP-3; proIL-1b; ICAM-1; a1-PI; galectin-3; plasminogen
Furin-activated Secreted MMPs		MMP-11	Stromelysin-3	Fn; Ln; aggrecan; gelatins	a1-PI; a2-M; IGFBP-1
		MMP-28	Epilysin	Unknown	Casein
Vitronectin-like insert MMPs		MMP-21	None	Unknown	Unknown

Table 1: Continued.

MMP group	MMP subgroup	Designation	Alternative name	Matrix substrate	Bioactive substrate
Minimal domain MMPs	Matrilysins	MMP-7	Matrilysin-1 (Pump-1)	Pro-α-defensins, FAS ligand, latent TNF, syndecan-1, E-cadherin, Elastin; Aggrecan; gelatin; Fn; Ln; elastin; entactin; collagens, III, IV, V, IX, X, XI; tenascin; decorin; link protein; vitronectin	Both lack the hemopexin-line domain, they process collagen IV but not collagen I, Pro a-defensin; Fas-L; b4 integrin; E-cadherin; proTNF-a; CTGF; HB-EGF; RANKL; IGFBP-3; plasminogen
		MMP-26	Matrilysin-2 (endometase)	Gelatin; collagen IV; Fn; fibrinogen; vitronectin	pro-MMP-9; a1-PI
Type I transmembrane MMPs	MTs-MMPs	MT1-MMP/MMP14		Pro-MMP-2, fibrillar collagens, Fibrin, Syndecan-1, γ1-subunit of laminin-5, collagen I, II, III; gelatin; aggrecan; Fn; Ln; fibrin; vitronectin; entactin; proteoglycans; Ln-5	Pro-MMP-2; Pro-MMP-13; CD44; MCP-3; tissue transglutaminase
		MT2-MMP/MMP-15		Fibrin Fn; tenascin; nidogen; aggrecan; entactin; collagen; gelatin; perlecan; Ln; vitronectin	Pro-MMP-2; tissue transglutaminase
		MT3-MMP/MMP-16		Fibrin, Syndecan-1; collagen III; aggrecan; gelatin; Fn; vitronectin.	Pro-MMP-2; tissue transglutaminase
		MT5-MMP/MMP-24		Gelatin; fibronectin; vitronectin; collagen, aggrecan; PG	Pro-MMP-2
GPI-linked MMPs	MTs-MMPs	MT4-MMP/MMP-17		Gelatin; fibrinogen	Unknown
		MT6-MMP/MMP-25		Gelatin; collagen IV; fibrin; Fn; Ln	ProMMP-2
Type II transmembrane MMPs	MTs-MMPs	MMP-23A	Femalysin	Unknown	Unknown
		MMP-23B		Gelatin	Unknown

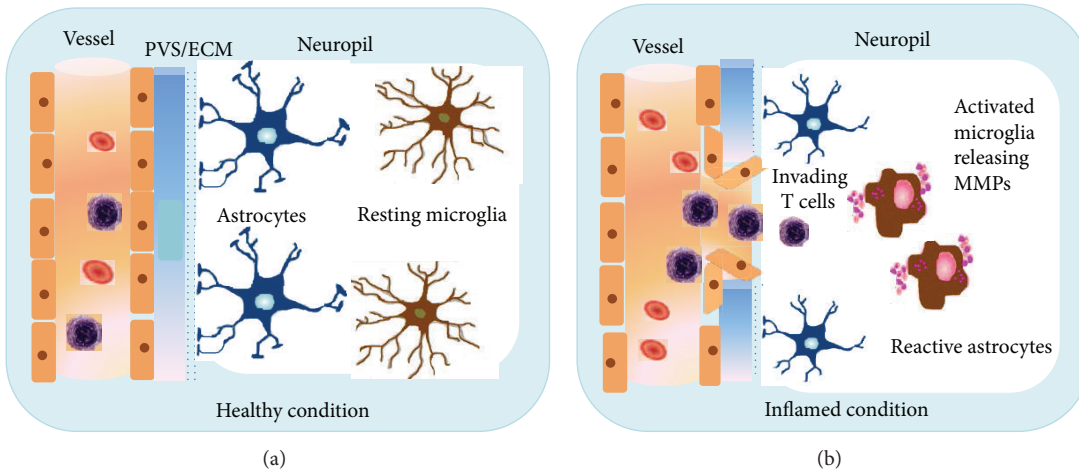

FIGURE 2: The blood brain barrier (BBB) in healthy and inflamed condition. (a) Vessel, endothelium, extracellular matrix (ECM), and glia limitans are intact. Microglia cells are in a resting state. (b) In the inflammed CNS breakdown of the BBB takes places. The glia limitans is opened, and astrocytic endfeet are drawn away. Reactive microglia secrete MMPs facilitating the opening of the BBB. Invading T cells migrate from the vessel via the perivascular space (PVS) into the neuropil.

P [53] and b-amyloid (1–40) [54], and MMP-2 cleaves b-amyloid (1–40) and b-amyloid (1–42) [55]. MMP-9 can also degrade human myelin basic protein (MBP), thereby directly contributing to myelin damage [56–58].

MMP-9 was called a tuner and amplifier of immune functions [59], because of its assistance in peripheralization of leukocytes in response to chemokines [59] into sites of inflammation and by acting as switch and catalyst at the interplay between the innate and adaptive immune systems. MMP-9 has been implicated in opening the route for immune cells into the neuropil in various diseases, including not only MS but also strokes and brain injuries [1, 25, 31, 60–65]. In fact, the infarct size can be lessened by reducing the MMP-9 activity with a monoclonal antibody [66] or through enzymatic inhibition respectively, gene knockout [65].

Although the cause of MS remains unknown, MMPs are implicated in the pathology of MS. Focal BBB leakage and extravasation of immune cells into the brain parenchyma are the earliest steps in the pathogenesis of MS as mentioned above [67, 68]. MMPs are effectors of BBB disruption [69]; extensive studies in MS and EAE demonstrated especially activity of MMP-2 [70] and MMP-9 [71, 72]. MMP-9 around blood vessels suggest that MMP-9 might be pathologically involved in the disruption of the parenchymal basement membranes [73], paving the way for infiltrating cells of the immune system [1]. Within the CNS immune cells orchestrate myelin and axonal destruction resulting in severe destruction of normal CNS constituents. The histopathological hallmark of MS is the plaque, a well-demarcated white matter lesion characterized by demyelination and axonal loss. Expression of MMP-1, -2, -3, -7, and -9 in monocytes/macrophages, microglia, astrocytes, and lymphocytes around perivascular cuffs in MS lesions has been described [73–75]. We could also confirm by immunostaining that microglia are sources of MMP-2 and MMP-9 (see Figure 3).

The secreted MMP-9 can cause demyelination and axonal injury [76, 77]. Axonal damage is considered to be a major cause of secondary progression (with irreversible neurological impairment) [78–80], which seems to be caused not only by T cells [81] but also by microglia/macrophages and their toxic products [75, 82, 83]. Cuzner et al. [84] could confirm enhanced MMP-9 expression in reactive microglia and astrocytes in autopsies from MS brains. Interestingly, intrathecal synthesis of MMP-9 appears to be specific for MS [85, 86]. Around the time of onset of the symptoms in EAE, elevated levels of MMP-9 can be found. The administration of GM6001 (a MMP inhibitor) improved the clinical condition by blocking the BBB injury [87].

The view that MMP-9 is a promoter of neuroinflammation has been additionally supported by the finding that young (3-4 weeks) but not older (7-8 weeks) MMP-9 null mice were less susceptible to development of EAE than wild type controls [71]. In addition, MMP-2 null mice were reported to have an earlier onset and more severe disease compared to wild type controls. Apparently this was related to a compensatory increase of MMP-9 in the MMP-2 null mice [71]. Enzyme inhibitors of MMPs have been shown to ameliorate the clinical course and reduce inflammatory cell infiltration in EAE [87–89]. Treatment of PTx-injected CCL2-overexpressing mice with the synthetic broad-spectrum inhibitor BB-94 (Batimastat) alleviated symptoms of neuroinflammation [90] and put blood-derived cells on hold in perivascular spaces. This was the first evidence that the second step of neuroinflammation, that is, passage of the glia limitans, but not the first, migration across the vascular wall, depends on MMPs.

The production of MMP-9 is negatively regulated by IL-4 [91], IL-10 [92], and interferon-β [93] whereas transforming growth factor-β was found to enhance the production of MMP-9 in transformed lymphocytes [94]. Furthermore it was shown that cytokines, chemokines [95, 96], eicosanoides and peptidoglycan, lectins, double-stranded RNS and endotoxin [31, 59, 65, 97, 98] are acting as soluble upregulators [99–101]. Potent stimulators of MMP-9 and MMP-2 expression in

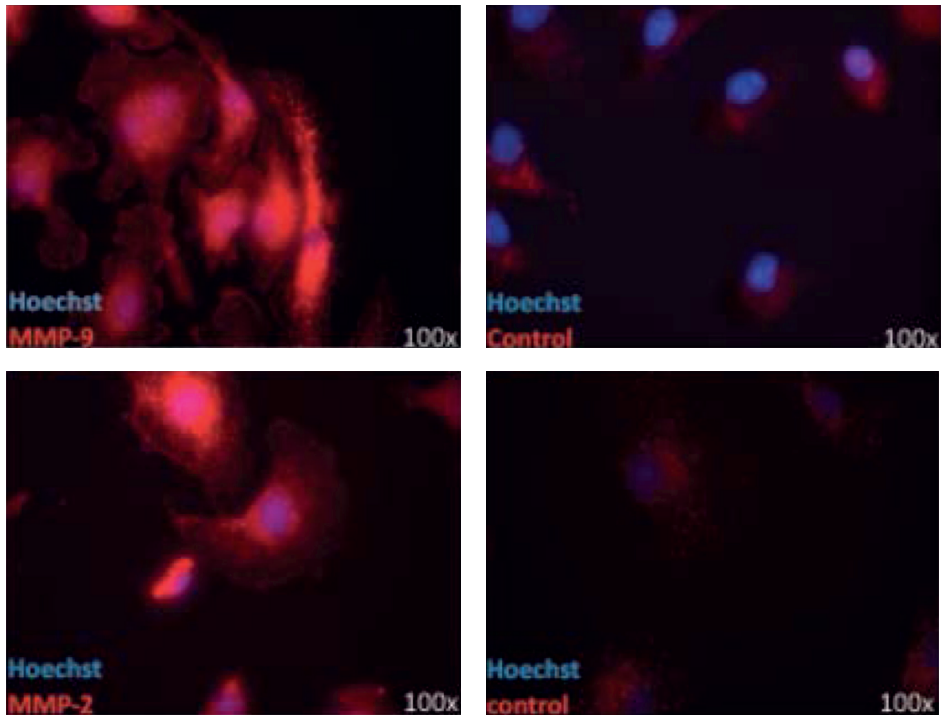

Figure 3: Immunohistochemistry of murine microglia, stained for MMP-2 and MMP-9. Microglia from CD11c GFP mice, fixed with PFA, treated with TBS and NGS, anti-MMP-2 (1 : 125) and anti-MMP-9 (1 : 500); control: BSA and secondary antibodies.

cultured astrocytes and microglia are the proinflammatory cytokines interleukin 1 (IL-1), tumor necrosis factor-alpha (TNF-α), and lipopolysaccharide (LPS) [102, 103].

Furthermore, interferon-β (an immune-modulator that is commonly used in MS) inhibits the expression of MMPs in glial cells. Liuzzi et al. [104] demonstrated that LPS treated microglia secreted higher levels of MMP-9. As soon as the microglia cells were pre-/treated with different doses of IFN-β they found dose-dependent inhibition of MMP-9. IFN-γ or IFN-β was also suggested to inhibit the expression of MMP-9 in human astroglioma and fibrosarcoma cell lines, as well as in primary astrocytes, supposable by the modulation of transcription factors that regulate the MMP-9 transcription [105, 106]. Still an indirect pathway cannot be excluded: IFN-β could regulate the MMP expression either through the reduction of proinflammatory cytokines or by the inhibition of the activity of enzymes involved in MMP activation [107]. IFN-β also reduces the production of MMP-9 by T cells and monocytes *in vitro* [8, 108, 109] leading to diminished MMP-9 levels in serum of multiple sclerosis patients [110, 111]. This was paralleled by the clinical recovery of the patients, presumably as a result of a significant reduction of T lymphocytes infiltrating in the brain. Besides interferon-β also increases gene transcription of TIMP-1, thus attenuating MMP overactivity in MS. Intravenous gamma globulins (IVIG) used in severe cases of MS were shown to diminish the amount of secreted MMP-9 and its mRNA expression [112].

In addition to their detrimental roles MMPs might also have a beneficial effect in MS, as they also have important functions in (the developmental) plasticity of the nervous system [70, 113, 114]: MMP-9 mediates the oligodendrocytes process outgrowth [115]. Cultured oligodendrocytes secrete MMP-9, and cell-associated gelatinases are found at the site of their growing tips of their processes [116].

Notably MMP-9 is significantly upregulated in the acute period of spinal cord injury [117, 118] which might promote the maturation of oligodendrocytes and their formation of myelin [119]. MMP-9 [115, 116] and MMP-12 [120] are expressed by oligodendrocytes and seem to be essential for regulating the extension of their processes. Remyelination was impaired in MMP-9 and MMP-9/-12-null mice, correlating with fewer mature oligodendrocytes [121]. Taking that into account the MMP-9 secretion by microglia might allow a microenvironment in lesions for better remyelination and repair [31]. MMP-2 levels increase between 7 and 14 days after spinal cord injury, and MMP-2 null mice do not recover equally well as wild type controls do suggesting that the delayed expression is necessary for ECM remodeling and functional recovery [122].

MMP-1, MMP-3, and MMP-8 were also reported to play a role in BBB disruption followed by a leukocyte infiltration into the brain [123, 124]. Woo et al. [125] demonstrated that the mRNA expression of MMP-1, -3, -8, and -9 in primary cultured microglia cells was significantly increased by LPS and other immunostimulants. Furthermore, the inhibition of MMP-3 and MMP-9 could suppress inflammatory reactions in activated microglia (such as iNOS, proinflammatory cytokine expression, and upstream signaling molecules such as MAPKs, which would amplify the inflammatory cascade by initiating the MMP production in an autocrine or paracrine way).

5. Conclusion

The data discussed beforehand reinforces the concept that leukocyte transmigration involves distinct molecular mechanisms. MMP-2 and MMP-9 are expressed by microglial cells which contribute to the formation of the glia limitans [126]. These cell types might be responsible for the opening of the glia limitans and the further progression of autoreactive immune cells into the neuropil. Besides the contribution of MMP-9 to BBB breakdown, it is also involved in the generation of autoimmune epitopes as well as the bioavailability of cytokines.

The treatment of neurological inflammation still remains a challenge today. Targeting MMPs in the CNS may serve as therapeutic option in autoimmune diseases. It is tempting to employ inhibitors of MMP activity to abrogate increased MMP expression (partially driven by microglial activation) within the inflamed CNS. However, MMPs also fulfill beneficial roles in the CNS, including mediation of tissue repair [31], synaptic plasticity [127], learning, and memory [128]. With respect to the multiple roles of MMPs, not only detrimental but also physiological, the need and the judicious application of specific inhibitors against individual MMPs should be highlighted. Due to their bifaced role, timing may also be crucial for therapeutic effects. There is the likelihood that nonspecific MMP inhibitors, although protecting against particular detrimental effects of some MMPs, could block useful actions of MMPs, thus slowing down disease recovery, too. It will be necessary to analyze further therapies aimed at decreasing MMP-2 and MMP-9 expression or activity.

6. Microglia and Gliomas

The most common brain tumors are malignant gliomas, infiltrating diffusely into normal brain parenchyma [129]. So far all current (multimodal) therapeutic approaches were ineffective, and life expectancy from the time of the diagnosis in glioblastoma multiforme is on average 14 months [130–133].

In 1921 Rio-Hortega [134], was the first to describe the presence of microglia cells in brain tumors. Microglia contribute substantially (at least 1/3) to the tumor mass of glioblastoma as they make up the largest population of tumor-infiltrating cells [135–138]. Microglia seem to possess a decisive tumor-supporting role by creating a microenvironment, which plays a critical role in tumor initiation and progression [139–144]. This special environment is also an immunosuppressive milieu, where, for example, IL-10 is released [135, 145, 146]. Microglia/macrophages play also an influential role in glioma invasion: there is a positive correlation in their density in gliomas with the invasiveness and grade of gliomas [139, 144]. The antitumor properties, namely glioma-cytotoxic effects of microglia, could only be shown *in vitro* so far [147, 148]. Favoring gliomas' growth could be due to a suppression and/or control of microglial cells by glioma cells and glioma-derived molecules (e.g., their loss of phagocytic function [149]. It was also suggested that, under the influence of glioma cells, microglia develop a different, noninflammatory phenotype suppressing their defense properties [139, 144, 150]. Instead of releasing proinflammatory cytokines, microglia upregulate enzymes that facilitate tumor invasion and proliferation. A key mechanism in the expansion and invasion of gliomas is the degradation of extracellular matrix by membrane-bound or secreted proteases such as MMPs [151], especially matrix-metalloproteinase-2 [152] and MMP-9 [151].

7. MMPs and Glioma Cells

Due to their ECM-degrading ability and confirmed upregulation in almost all cancer entities, MMP-2 has been linked to invasiveness and dissemination [153–155]. Because serum concentration of MMP-2 was significantly elevated in tumor patients, MMP-2 was suggested as a diagnostic and prognostic marker [156, 157]. On the other hand elevated MMP-9 levels in the serum seem to be even more relevant values, because in healthy individuals under physiological conditions MMP-9 is hardly detectable [158]. Abnormal MMP-9 concentrations in patients serum were also shown for brain cancers [159], and notably there is a positive correlation with poor prognosis [160].

So far, there is no evidence that links MMP-2 to a special phase of tumor development (in contrast to MMP-9): besides creating a microenvironment in the early phase favoring cancer growth (activation of growth factors), the transition into an undifferentiated phenotype permitting migration and invasiveness is also related to MMP-2 activity, for example, the proteolytic detachment of adhesion molecules like integrins or cadherins or cytoskeleton changes [161, 162]. MMP-2 acts in multiple ways on tumor cells by modulation of their metabolism, their receptor turnover [163], and their resistance to apoptosis [164]. In fact, anti-MMP-2 siRNA-treated glioma cells underwent apoptosis [165] and MMP-2 inhibition autophagy-associated cell death [166].

The expression of the MMP-2 gene in human glioma tissues was found to be upregulated in comparison to normal brain tissue, and dramatically increased in glioblastomas [167–169]. MMP-9 expression could be correlated with high malignancy and progression of gliomas [170, 171]. Various studies show that glioma and microglia cells both produce MMP-2 *in vitro* [144, 168] and *in situ* [172]. However, MMP-2 is released as an inactive profrom by glioma cells (especially at the invasive tumor zone), and glioma cells themselves are unable to activate pro-MMP-2. Since the extracellular activator MT1-MMP is highly upregulated in glioma infiltrating microglia [138, 151, 172, 173], glioma cells engage microglial cells to promote their spread and survival [174]. This concept of microglial "abuse" has been impressively demonstrated by the group of Kettenmann [138].

8. Pathways of MMP Induction and Suppression in Gliomas

Another key player of glioma motility and invasion seems to be FasL, which is expressed in tumor cells. It not only induces apoptosis in T cells thereby leading to local immunosuppression, but blockade of Fas signaling resulted in impaired

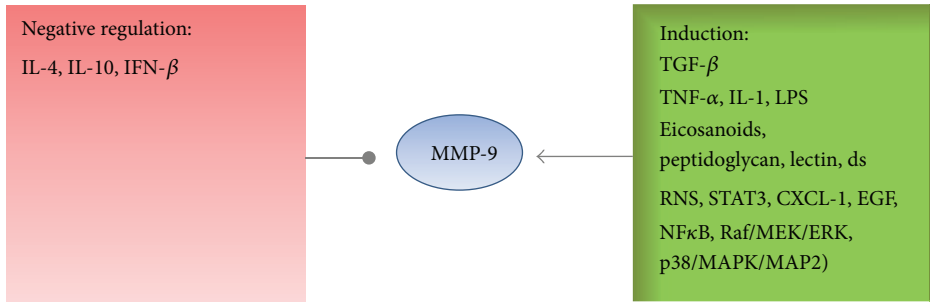

Figure 4: Regulation and induction of MMP-9: a variety of molecules are involved.

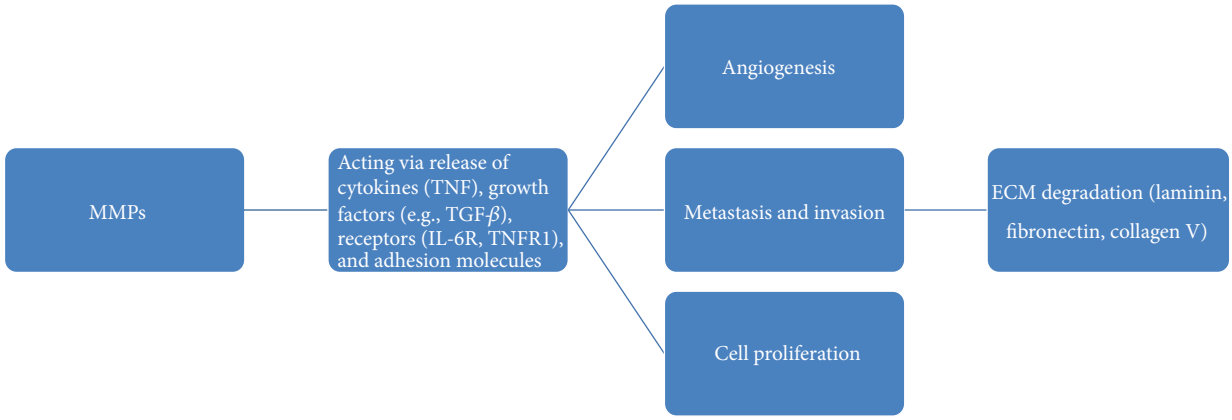

Figure 5: MMP in gliomas: the various roles of MMPs in promoting the growth of cancer cells.

MMP-2 activity with a subsequent reduction of glioma invasiveness and motility [175]. The expression of MMPs is also facilitated by glioma-derived TGF-β which suppresses the expression of TIMPs and also has an immunosuppressive role [176–178]. The inactive precursor of TGF-β can be processed by MMP-2 [179, 180], and TGF-β induces gene transcprition of MMP-2, thus generating a vicious circle leading to further tumor growth [181, 182] (Figure 4).

Another player in glioma growth, gliomagenesis, and progression is the activation of STAT3 [183]. This signal transducer and activator of transcription protein 3 is constitutively activated in glioblastoma cell lines [184–187] and increases MMP-9 expression and activation in human astrocytoma cell lines [188, 189]. The expression of MMP-2, -9, and -14 in microglia/macrophages was also shown to be enhanced by glioma-derived CX3CL1 (Chemokine (C-X3-C Motif) Ligand) and is significantly associated with the recruitment of microglia into the tumor [190]. Another role in the production of MMP-9 in glioma cells is played by protein kinase C (PKC) [191] and IL-6 is a confirmed growth factor for glioma stem cells, too [192]. The tumor-promoting role of IL-6 may be exerted via MMP-2, whose production is increased by IL-6 [193]. Glioma-induced MMP-2 activity in microglia could be significantly decreased by the A1AR (an adenosine receptor subtype, found on microglia and neurons) [194], which might explain the fact that adenosine treatment leads to decreased extracellular protease activity and thereby exerts its inhibitory effects on glioma invasion. Early studies in MS patients could also show that A1AR activation in microglia interfered with the MMPs production [195].

Although the substrate specifity of MMP-2 and MMP-9 overlaps, MMP-9 (in contrast to MMP-2) is highly inducible mostly by integrins, growth factors, and cytokines [13, 196] leading to a defined chronologically and spatially distribution. The expression of MMP-9 is further triggered by autocrine or paracrine mechanisms (IL-1β, TNF-α, and TGF-β), cell binding (to fibronectin or vitronectin), EGF release or distinct molecular pathways (transcription factors NF-κB, Raf/MEK/ERK cascade, or the p38 MAPK/MAPK2-signaling) [180, 196–200] (Figure 5).

In sum, ample data describe the communication between tumor cells and microglia. Microglia and their expression of MMPs could be a crucial target for future therapeutic options in gliomas, due to their profound involvement in tumor progression.

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Review Article

The Controversial Role of Microglia in Malignant Gliomas

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Malignant gliomas contain stroma and a variety of immune cells including abundant activated microglia/macrophages. Mounting evidence indicates that the glioma microenvironment converts the glioma-associated microglia/macrophages (GAMs) into glioma-supportive, immunosuppressive cells; however, GAMs can retain intrinsic anti-tumor properties. Here, we review and discuss this duality and the potential therapeutic strategies that may inhibit their glioma-supportive and propagating functions.

1. Introduction

Microglia can constitute up to 10% of cells in the central nervous system (CNS) and are distinctive from other CNS cells such as astrocytes and oligodendrocytes [1]. Distinguishing features of microglia are their "ramified" branches that emerge from the cell body that communicate with surrounding neurons and other glial cells. Microglia rapidly respond to infectious and traumatic stimuli by adopting an "amoeboid" activated phenotype and can produce a variety of pro-inflammatory mediators such as cytokines, chemokines, reactive oxygen species (ROS), and nitric oxide (NO), which contribute to the clearance of pathogenic infections. However, prolonged or excessive activation may result in pathological forms of inflammation that contribute to the progression of neurodegenerative and neoplastic diseases [2].

Gene transcript clustering analysis reveals that microglia have close lineage relationship with bone-marrow-derived macrophages, indicating that these cells arise from the bone marrow and circulating monocytes/macrophages [3]. However, recent findings suggest that microglia originate from yolk sac macrophages that migrate into the CNS during early embryogenesis [4] (Figure 1). Further confounding the issue regarding the origin of CNS microglia are studies demonstrating the generation of microglia-like cells from a murine embryonal carcinoma cell line (P19) during neural differentiation [5]. Regardless of the origin, the microglia/macrophage population are usually the dominant glioma-infiltrating immune cells (5–30%) [6].

Studies during the 90s demonstrated a positive correlation between the number of microglia/macrophage and glioma malignancy [7, 8]. Furthermore, microscopic analysis of microglia morphology in high-grade glioma revealed an activated state, described by amoeboid or spherical shape [9, 10]. Morioka et al. have found that reactive microglia form a dense band that surround the tumor mass and can extend along the corpus callosum into the contralateral cerebral hemisphere [11]. These data would indicate that microglia react to brain tumors; however, it remains to be determined whether this response represents an active anti-tumor defense mechanism or a tumor-supportive one. Likewise, microglia are commonly found "trapped" in gliomas and it is unclear if they have coproliferated with the tumor cells. Hepatocyte growth factor (HGF)/scatter factor (SF), which plays a role in glioma motility and mitogenesis, may be one chemokine responsible for the microglia infiltration in malignant gliomas [12]. Additionally or alternatively, monocyte chemotactic protein-3 (MCP-3) was found to correlate with GAM infiltration [13] and to act as a chemokine. Furthermore, it has been reported that tumor necrosis factor (TNF) dependent action enhances macrophage/microglia recruitment in glioma [14]. Other chemoattractants that have been shown to stimulate microglia/macrophage migration into the tumor include colony stimulating factor-1 (CSF-1) [15, 16], macrophage colony-stimulating factor (M-CSF) [17], and glial-derived neurotrophic factor (GDNF) [18]. However, recent studies have emphasized a predominant role of

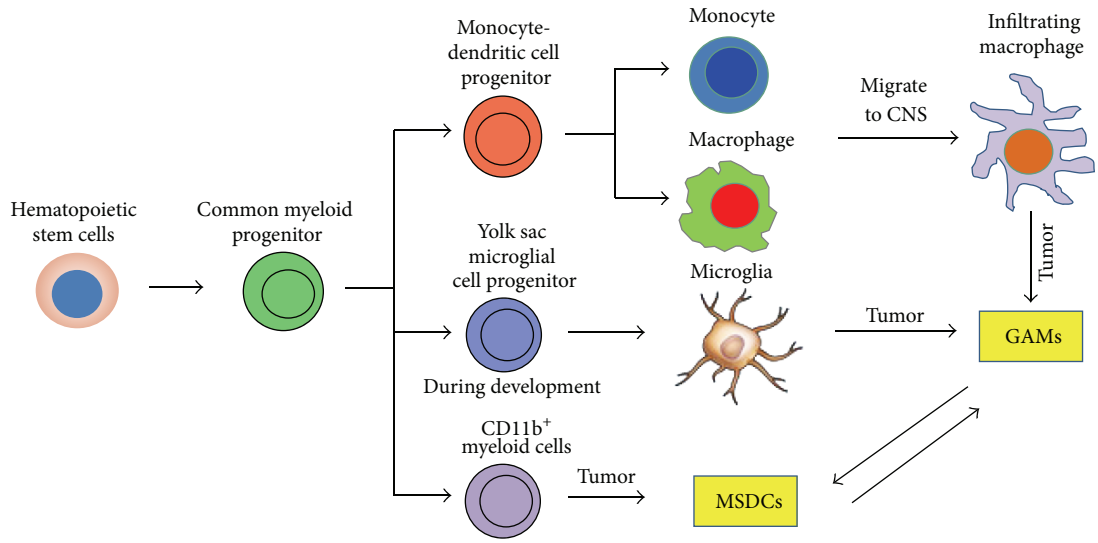

Figure 1: Cell lineage derivation of the CNS microglia/macrophage is depicted, with arrows indicating lineage relatedness. Myeloid-derived suppressor cells (MDSCs) are a lineage term describing glioma-associated microglia/macrophages (GAMs).

granulocyte-macrophage colony stimulating factor (GM-SCF) in microglia/macrophage attraction [19] which confirmed previous reports [20, 21]. Nonetheless, the increased number of microglia/macrophages found in high-grade gliomas has suggested that they may have a pro-neoplastic role [8].

No consensus definitive marker that distinguishs between microglia and macrophages has thus far been defined. As such, many investigators use the more general term "microglia/macrophages" instead of microglia alone. CD163, CD200, CD204, CD68, F4/80, and the lectin binding protein Iba-1 can be used as general markers of microglia/macrophages [22, 23]. P2X4R expression can define a distinct subset of GAMs [24]. Furthermore, allograft inflammatory factor-1 (AIF-1) and heme oxygenase-1 (HO-1) can also be used to define a distinct subset of GAMs in rat and human gliomas [25, 26]. However, the most commonly used criterion to distinguish CNS microglia from macrophages is the differential CD45 expression ($CD45^{low}$ for microglia and $CD45^{high}$ for macrophages) on $CD11b^+$ $CD11c^+$ cells [27–29]. The robustness of this is questionable.

Another confounding issue in defining and clarifying the biological role of GAMs is their distinction from myeloid-derived suppressor cells (MDSCs). The MDSCs are induced in response to various tumor-derived cytokines and have been shown to inhibit tumor-specific immune responses [30]. However, this is not an absolute since MDSCs isolated from mouse brain tumors, although expressing markers consistent with an immune suppressive phenotype (CCL17, CD206, and CD36), still express proinflammatory IL-1β, TNF-α, and CXCL10 [31]. Currently, there are two presumed MDSC populations: monocytic and granulocytic. Thus far little is known about the biology and phenotypic characteristics of MDSCs within gliomas but presumptively GAMs would be closer in the continuum to the monocytic MDSC subset based on $CD11b^+$ expression and function. Specifically, MSDCs only weakly present glioma antigens to cytotoxic T cells and express FasL, which contributes to the local immunosuppressive milieu of malignant gliomas [32], similar immunological functions attributed to GAMs [33]. Furthermore, the MDSCs have been shown to inhibit T cell activity by NO production [34]; however, this immune suppression was postulated to be distinct from CNS microglia.

2. The M1/M2 Continuum

The M1/M2 continuum has been applied to CNS infiltrating macrophage/monocytes in the context of inflammation or tumor. Classically activated macrophages assume a M1 phenotype characterized by the expression of the signal transducer and activator of transcription 1 (STAT-1) and the production of iNOS (Figure 2). The M1 cell is capable of stimulating anti-tumor immune responses by presenting antigen to adaptive immune cells, producing pro-inflammatory cytokines and phagocytosing tumor cells [35] (Figure 3). Whereas the alternatively activated pathway, M2, is characterized by the expression of surface CD163 and CD204, expression of intracellular STAT-3 and the production of arginase [36, 37] (Figure 2). M2 polarization prevents the production of cytokines required to support tumor-specific $CD8^+$ T cells, and $CD4^+$ T helper 1 (Th1) and promotes the function of $CD4^+$ regulatory T cells, and are therefore tumor supportive [38, 39] (Figure 4). Multiple lines of evidence suggest that the GAMs are likely skewed to the alternatively activated M2 macrophage phenotype. However, to date, it should be noted that a comprehensive characterization of the M1 and M2 composition of gliomas has not been conducted.

Glioma cells secrete a wide variety of factors that suppress immune cells, such as IL-10, IL-4, IL-6, M-CSF, macrophage inhibitory factor (MIF), TGFβ, and prostaglandin E2 (PGE2) [17, 40–43]. These cytokines are known to promote a M2 phenotype and/or to suppress the M1 phenotype. For example,

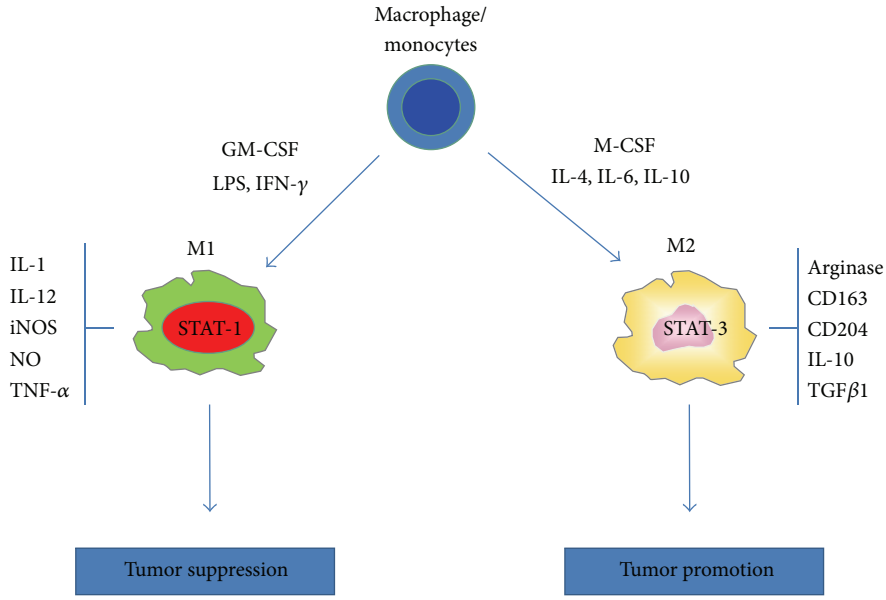

Figure 2: CNS macrophage/monocytes differentiate into polarized macrophage subsets when exposed to different cytokine milieu. In the presence of granulocyte-macrophage colony stimulating factor (GM-CSF), interferon-(IFN) γ, lipopolysaccharide (LPS) and other microbial products, monocytes differentiate into M1 macrophages. In the presence of macrophage colony stimulating factor (M-CSF), interleukin-(IL) 4, IL-6, IL-10 and immune suppressive molecules (corticosteroids, vitamin D3, prostaglandins), monocytes differentiate into M2 macrophages. M1 and M2 subsets differ in terms of phenotype and functions. M1 cells have high anti-microbial activity, immune stimulatory functions and tumor cytotoxicity and express the signal transducer and activator of transcription 1 (STAT-1). M2 cells have high scavenging ability, promote tissue repair and angiogenesis, favor tumor progression and express STAT-3.

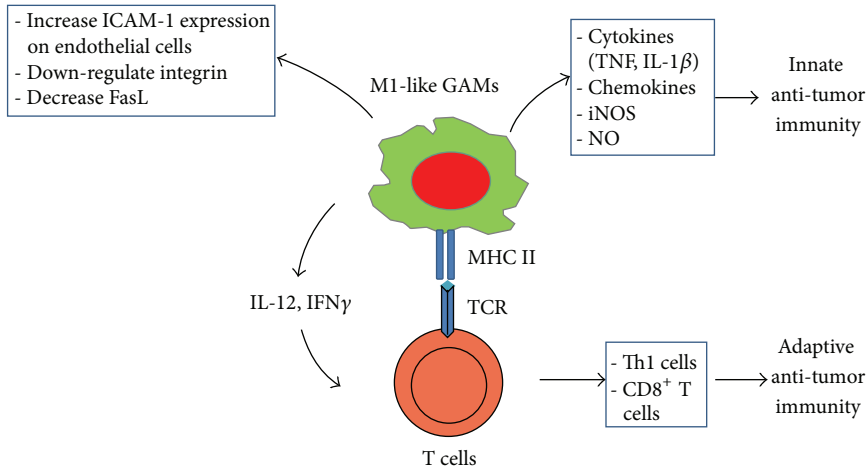

Figure 3: Glioma-associated microglia/macrophages (GAMs) have anti-tumoral potential. In certain circumstances, GAMs can be activated and polarized to a M1-like phenotype that can contribute to both innate and adaptive anti-tumor immunity.

TGFβ inhibits microglia cell proliferation and the production of proinflammatory cytokines *in vitro* [44]. IL-4, IL-6 and IL-10 have been shown to polarize microglia to an M2-like phenotype [45]. Other immunosuppressive mechanisms such as the downregulation ICAM-1 and expression of immune inhibitory molecules such as B7-H1 can also dismantle the microglia-T cell combined immune recognition and clearance of gliomas [46]. Furthermore, gliomas induce upregulation and expression of HLA-G and HLA-E by GAMs in a majority of glioblastomas, thus hindering anti-glioma activity [47]. The anti-glioma functional impairment of GAMs likely occurs relatively late in the course of glioblastoma tumor growth, potentially providing a window of opportunity for therapeutic strategies directed towards preventing their functional impairment [48]. Of note, the M1/M2 distinction is a continuum and simplification of the complex T cell and GAM interactions and the subsequent outcome.

Similar impairments of microglia/macrophage anti-tumor activity have been reported for brain metastasis. In the region where the tumor mass is situated, only a few microglia

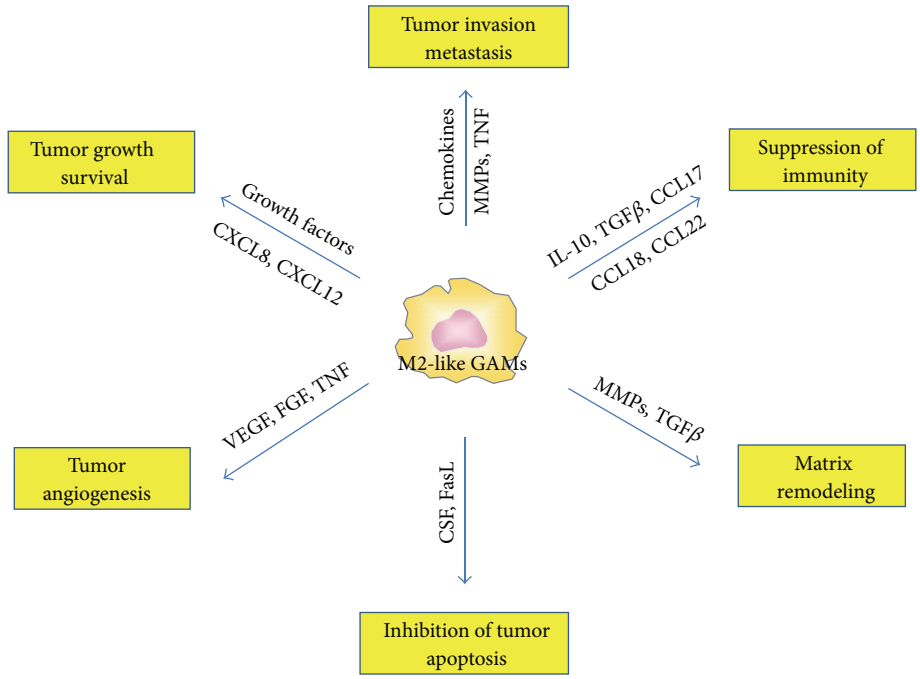

Figure 4: Glioma-associated microglia/macrophages (GAMs) are tumor supportive. Chemokines have a prominent role as they induce neoangiogenesis, activate matrix-metalloproteases (MMPs) and stroma remodeling, and direct tumor growth. Selected chemokines and immunosuppressive cytokines inhibit the anti-tumor immune response.

express inducible nitric oxide synthase (iNOS) and tumor necrosis factor-alpha (TNF-α). On one hand, the microglia accumulate as a result of migration and proliferation, have an ameboid appearance, and appear to actively respond to the metastatic lung cancer cells in the brain by tightly encapsulating the tumor mass. On the other hand, the lack of positivity of either iNOS or TNF-α in double-labeling experiments indicates that the microglia immunological functions including cytotoxicity, phagocytosis, and antigen presentation are impaired [49]. Microglia have also been shown to promote invasion and colonization of the brain by breast cancer by serving both as active transporters and guiding rails. This is antagonized by inactivation of microglia as well as by Wnt signaling inhibition. Therefore, microglia were shown to be critical for the successful colonization of the brain by epithelial cancer cells, suggesting that inhibition of microglia could also be a promising anti-metastatic strategy [50].

Despite the immunosuppressive environment of human gliomas, GAMs are capable of some innate immune responses such as phagocytosis, cytotoxicity and TLR expression but are not competent in secreting key pro-inflammatory cytokines [51]. GAMs responsiveness to activators is impaired when compared to microglia/macrophages isolated from normal brain; specifically, the former has an impaired capacity to be stimulated by TLR agonist to secrete cytokines, upregulate costimulatory molecules, and activate anti-tumor effector T cells [33]. Understanding the mechanism of these differences may be critical in the development of novel immunotherapies for malignant gliomas [52].

The GAMs do not always display MZ functions. Insoluble matrix components derived from malignant glioma cells have been shown to enhance microglia activation [30]. These activated microglia can then induce glioma cell death by blockade of basal autophagic flux inducing secondary apoptosis/necrosis [53]. Signaling through the JAK-2-STAT-5 pathway was shown to be essential for IL-3-induced activation of microglia [54]. Furthermore, microglia/macrophages depletion increased glioma tumor volume by 33%. This was not believed to be secondary to the loss of microglia tumoricidal activity because phagocytosis or apoptosis of glioma cells was rarely observed. The loss of anti-tumor effect was also not due to alterations in tumor vasculature. Rather, the investigators found that depletion of a subset of $CD86^+$ microglia with M1-like characteristics could present alloantigen and secrete stimulatory cytokines that promoted the expansion of the $CD8^+$ T cells and was likely the etiology for enhanced glioma growth [55].

Alternatively, M2-like GAMs also promote glioma growth/survival via enhancing angiogenesis and inhibiting tumor apoptosis. GAMs have been shown to release growth factors such as VEGF, PDGF, and members of the FGF family, and a correlation has been shown between GAM numbers and tumor vascularity in gliomas [56]. Furthermore, these growth factors sustain malignant cell survival and subsequent tumor growth. Lastly, GAMs are a major source of FasL expression, which likely contributes to the suppression of malignant glioma apoptosis [32].

Glioma regression has also been correlated with greater numbers of T cells and microglia, suggesting that the combined mobilization of peripheral and CNS endogenous immune cells is required for eradicating large intracranial tumors [57]. Some soluble factors from reactive microglia are capable of enhancing the expression of ICAM-1 on the brain

endothelial cells (ECs). As a consequence, large numbers of tumor-primed T lymphocytes can adhere to EC and migrate across the EC monolayer [58]. Finally, antibody-dependent cell mediated cytotoxicity (ADCC) has also been shown to be involved in microglia anti-tumor activities. Microglia derived from brain cortices of newborn mice were shown to lyse human tumor cell lines expressing different levels of epidermal growth factor receptor (EGFR) in the presence of a monoclonal antibody specific to EGFR [59]. Reconciliation of the cumulative data would indicate that GAMs can be either M1 or M2 or some were in between, but the functional outcome will depend on the relative composition of M1 and M2 cells within the glioma.

3. Interplay between GAMs and Gliomas

It is becoming apparent that crosstalk exists between the GAMs and the brain tumor cells. Gliomas promote the recruitment, proliferation, and M2 polarization of microglia/macrophages; reciprocally, GAMs facilitate the survival, growth, and especially the spread of glioma cells (Figure 4). When activated in the presence of glioma, microglia invade the tumor. The microglia then secrete a variety of factors that degrade the CNS matrix. The glioma cells simultaneously invade and expand into the dissociated tissue by utilizing the same corridor paved by laminin on astrocytic projection for microglia invasion into the tumors [60–62]. Glioma secreted factors, by engaging the toll-like receptors and the p38 MAPK pathway, trigger the expression and activity of membrane type 1 metalloprotease (MT1-MMP) on GAMs. The GAM MT1-MMP expression then in turn activates glioma-derived pro-MMP-2 that subsequently promotes glioma invasion. A deficiency of MyD88, an upstream mediator of MT1-MMP, or microglia depletion, largely attenuates glioma expansion *in vivo* [62]. Furthermore, in brain slices inoculated with glioma cells, increased activity of metalloprotease-2 was directly correlated with the abundance of microglia [63]. Another GAM-associated mechanism, the CX3CR1/CX3CL1 interaction, can also induce MMPs production resulting in glioma invasion [64]. A recently described factor, STI1 (cochaperone stress inducible factor 1) secreted by microglia was shown to favor tumor growth and invasion through the participation of MMP-9 [65]. Thus, glioma cells stimulate microglia to increase the breakdown of extracellular matrix, thereby, promoting glioma invasion.

Substantially more controversial is whether microglia initiate or participate during early gliomagenesis. Neurodegeneration, neurotoxicity, and neuroinflammation are associated with chronic microglia activation that has been postulated to contribute to gliomagenesis [66–68]. Cyclooxygenase-1 (COX-1) in microglia/macrophages might represent a key regulatory mechanism in the inflammatory processes associated with neoplasia [69]. COX-1 and COX-2 differential accumulation is observed in microglia/macrophages and astrocytes during oligodendroglioma progression *in vivo* [70]. Furthermore, induction of COX-2 in microglia contributes to the deleterious effects of prostanoids in cerebral edema formation during the progression of oligodendrogliomas [71]. Increased c-Jun-NH2-kinase signaling in neurofibromatosis-1 heterozygous ($Nf1^{+/-}$) microglia was shown to promote optic glioma proliferation [72] and glioma growth [73]. Interestingly, IL-4 or IFN-γ-mediated microglia activation differentially induces oligodendrogenesis and neurogenesis, respectively, from adult stem/progenitor cells. It thus appears that how microglia are activated determines their ability to either support or impair cell renewal and differentiation from adult stem cells [74].

Glioblastomas are believed to arise from glioma stem cells (GSCs). GSCs are phenotypically similar to normal stem cells, can express CD133, and possess self-renewal potential [75]. GSCs recapitulate the original polyclonal tumors when xenografted into nude mice and mediate chemo- and radiation resistance, thereby, leading to tumor progression and recurrence. A positive correlation is found between the degree of infiltration of GAMs and the density of GSCs. The capacity of GSCs to recruit GAMs was found to be stronger than glioma cell lines indicating that the GSCs play a predominant role in microglia/macrophages tropism to glioma [76]. In addition, recent mechanistic studies by another group showed that TGFβ1 released by GAMs promoted the expression of MMP-9 by GSCs, and TGFR2 knockdown reduced the invasiveness of these cells *in vivo* [77].

We have previously shown that GSCs produce a variety of cytokines known to recruit and polarize the microglia/macrophages to become immunosuppressive. The GSC-conditioned medium polarized the microglia/macrophage to an M2 phenotype, inhibited microglia/macrophage phagocytosis, induced the secretion of the immunosuppressive cytokines IL-10 and TGFβ1 and enhanced the capacity of these cells to inhibit T-cell proliferation [44]. The inhibition of antigen-presenting capabilities of GAMs by glioma tumor cells has also been demonstrated [25]. Previously, glioma-derived M-CSF was shown to induces markers reflective of the M2 phenotype CD163 and CD204 on monocytes, and in turn these differentiated microglia/macrophage facilitated tumor growth. Both the extent of M-CSF production and $CD163^{+}$ and $CD204^{+}$ expression on microglia were correlated with glioma grade [17]. A direct correlation has been shown between the immunogenicity of glioma tumor cells and the GAM content and their antigen-presenting function [78, 79]. In addition, the metabolic status of the microglia is altered by the glioma environment [80]. Abundant amounts of ATP released by the glioma have been suggested to trigger $P2X_7R$ signaling, resulting in increase of MIP-1α (macrophage inflammatory protein-1α) and MCP-1 (monocyte chemoattractant protein-1) in GAMs [81]. Cumulatively these data indicate that there is a strong interaction that occurs between the GAM and glioma that ultimately influences the biological behavior of each one.

4. Therapeutic Manipulation of the GAM

A variety of therapeutic anti-glioma strategies have suggested that modulation of the GAM population may contribute to therapeutic efficacy. Inhibition of experimental rat glioma

Table 1: Microglia activating agents.

Molecule/agent	Classification	Mechanism/action	Reference
CpG-ODN	TLR9 ligand	Increases microglia tumor infiltration and enhances the antigen-presenting capacity	[87–89]
poly (I:C)	TLR3 ligand	Unknown soluble factors	[90]
IL-12	Th1 cytokine	Increases tumor infiltration and enhances TRAIL and phagocytosis	[91, 92]
TNF	Th1 cytokine	Enhances glioma cytotoxicity	[93]
IFN-γ	Th1 cytokine	Upregulates class II MHC antigen expression	[94]
Cytotoxic T cells	Immune cells	Induce microglia activation and recruitment	[95, 96]
C1q, complement receptor 3 (CR3)	Complement	Mediates elimination of tagged synapses and activates microglia	[97, 98]
T11TS/SLFA-3	Glycopeptide	Induces MHC class II expression and facilitates SLFA3/T11TS-CD2 immune activation	[99, 100]
Ceramide	Sphingolipid	Enhances microglia production/secretion of brain-derived neurotrophic factor (BDNF)	[101]
Ganglioside	Glycosphingolipid	Activates microglia via protein kinase C and NADPH oxidase, which regulate activation of NF-κB	[102]
Adenosine	Nucleoside	Acts via A1 adenosine receptors in microglia	[60]
Triggering receptor expressed on myeloid cells-2 (TREM2)	Innate immune receptor	Increases phagocytosis	[103]
Prothrombin	Blood-clotting protein	Activates microglia via kringle-2 domain	[104]
Propentofylline (PPF)	Methylxanthine	Inhibits microglia migration toward tumor cells and decreases MMP-9 expression	[105]
Minocycline	Antibiotic	Reduces glioma expansion and invasion by attenuating microglia MT1-MMP expression	[106]
Cyclosporin (CsA)	Immunosuppressant	Inhibits immunosuppressive microglia via MAPK signaling	[107]
Mifamurtide	Muramyl dipeptide	Enhances macrophage cytotoxicity and has been used for osteosarcoma treatment	[108]
Butyrate	Fatty acid	Anti-inflammatory in primary, brain-derived microglia cells, but is proinflammatory in transformed, proliferating N9 microglia	[109]
I-125	Radioactive isotope	Stimulates microglia/macrophage to remove necrotic debris	[110]

growth by decorin (TGFβ antagonism) gene transfer was associated with decreased microglia infiltration suggesting that the GAMs were participate in the regression of decorin-expressing rat C6 gliomas [82]. Recent studies have demonstrated that ablation of CD11b$^+$ cells in ganciclovir-treated CD11b-HSVTK mice [83] or *in vivo* targeting folate receptor β (FRβ)-expressing tumor-associated macrophages, decreases tumor size and improves animal survival [84]. The presence of significant anti-tumor immunity following herpes simplex virus 1 thymidine kinase (HSV-TK) and ganciclovir (GCV) treatments suggests that the immune system plays a critical role in the sustained tumor regressions associated with these treatments. Histologic examination of the brains of the successfully treated animals demonstrated residual tumor cells and inflammatory cells consisting predominantly of macrophages/microglia and T cells [85]. Another report demonstrates that a reduction of peripheral CD163$^+$ macrophages *in vivo* and the depletion of CD68$^+$ macrophage/microglia within brain slice *ex vivo* increase the intratumoral oncolytic viral titer to 5-fold and 10-fold, respectively, [86].

Therapeutic targeting of the GAM could be directed toward their activation (Table 1). Clearly, the goal of therapeutically targeting GAMs would be to selectively inhibit M2 while enhancing M1 functions (Table 2). The latter approach would include strategies that (1) inhibit the molecular mechanisms used by M2 cells to block lymphocyte reactivity and proliferation; (2) induce M2 apoptosis and/or trafficking to the tumor; or (3) force GAMs to the M1 phenotype. Inhibition of the M2-like activation of tumor-infiltrating macrophages was shown to significantly reduce glioma growth [37]. As such, STAT-3 is an attractive candidate since this pathway mediates glioblastoma-mediated M2 skewing and immune suppression. STAT-3 blockage by WP1066 stimulates the immune activation of GAMs, as evidenced by their increased expression of costimulatory molecules CD80 and CD86 [117]. Furthermore, *in vivo* STAT-3 inhibition in murine GAMs was shown to reduce expression of immunosuppressive cytokines, such as IL-10 and IL-6, while stimulating production of pro-inflammatory TNF-α [118]. Another therapeutic option is glycoprotein T11TS, which has been found to upregulate MHC class II, CD2 and

Table 2: Molecules/targets in GAMs for therapeutic modulation.

Molecule/agent	Classification	Mechanism	Reference
CSF-1R	Cytokine receptor	Inhibits glioblastoma invasion by targeting glioblastoma-associated microglia via inhibition of the CSF-1R	[16, 111]
TGFβ1	Cytokine	GAMs enhance the invasion of GSCs via TGFβ1 signaling pathway, which increases the production of MMP-9 by GSCs	[77, 112]
IL-4	Cytokine	Inhibits inflammatory mRNA expression in mixed rat glial and in isolated microglia cultures	[113]
IL-16	Cytokine	Expression correlates with WHO grades of human astrocytic brain tumors	[114]
MCP-1	Cytokine	A positive amplification circuit for macrophage recruitment in gliomas	[115]
Vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activating polypeptide (PACAP)	Neuropeptides	Inhibit the production of inflammatory mediators by activated microglia, thus, defined as "microglia-deactivating factors"	[116]
STAT-3	Transcription factor	STAT3 inhibition activates tumor macrophages and abrogates glioma growth	[44, 117–119]
Cyclophosphamide (CPA)	Alkylating agent	Pretreatment with CPA inhibits an increase of $CD68^+$ and $CD163^+$ cells and therefore enhances HSV replication and oncolysis	[120]
Dexamethasone	Steroid	Inhibits the filtration of microglia into brain tumors	[121]
ATP	Nucleotide	Promotes an anti-inflammatory state in both hematogenous and resident myeloid cells of the CNS	[122]
Radiochemotherapy	Therapy	Depletes $CD68^+$ microglia	[123]

CD4 expression in microglia *in vivo* in a rat glioma model [40]. Because macrophages/microglia express the nicotinic acetylcholine receptor (AchR) on their surface, a short AchR-binding peptide derived from the rabies virus glycoprotein (RVG) could also be used for targeted delivery of siRNA to macrophages for anti-tumor treatment. This peptide was fused to nona-D-arginine residues (RVG-9dR) to enable siRNA binding [124]. Recently, GAMs were shown to enhance GSCs' invasion via the TGFβ1 signaling pathway [77]. shRNA against TGFβ1 receptor (TGFβR) on tumor-associated macrophages strongly inhibited glioma invasiveness, indicating that TGFβR is a potential therapeutic target [112]. Several small molecules exist with TGFβR inhibitor activity that could be utilized as GAM modulatory agents. Studies of Carpentier et al. [87] revealed that intratumoral injections of oligodeoxynucleotides containing CpG motifs (CpG-ODN) trigger both innate and specific immunities, driving the immune response towards the Th1 phenotype. On the other hand, glioma-bearing animals treated with minocycline showed increased survival [125] and reduced glioma invasion by attenuated microglia MT1-MMP expression [106].

It should be noted that glioblastoma-mediated immune suppression is notoriously heterogeneous and plastic. Thus, targeting the M2 population may only result in a therapeutic response in a subset of patients in which this mechanism is operational. Furthermore, the therapeutic response of M2 targeting may be limited when other immune suppressive mechanisms are appropriated or upregulated by the tumor. However, the suppression/inhibition of glioma-derived factors or glioma-mediated immune suppression has been shown to synergize with the efficacy of microglia therapeutic strategies [118, 126–129], suggesting that there is a therapeutic opportunity.

GAMs or their precursors could be used to facilitate CNS tumor imaging [130]. Accurate delineation of tumor margins is vital to the successful surgical resection of brain tumors and the extent of resection impacts survival. The nanoparticle CLIO-Cy5.5 is taken by microglia and is detectable by both magnetic resonance imaging and fluorescence. It could be used to assist intraoperatively in visualizing tumor boundaries because $CD11b^+$ microglia are found at the tumor margin [131]. Other labeling system include cyclodextrin-based nanoparticles (CDP-NPs) [132] or multiwalled carbon Nanotubes (MWCNTs) [133]. Recently, quantum dots have been shown to be phagocytized by microglia and macrophages that infiltrate experimental gliomas that resulted in improved identification and visualization of tumors, potentially augmenting brain tumor biopsy and resection [134]. Ultimately, these imaging approaches could be exploited as biomarkers to monitor clinical trials targeting the GAM population [135].

5. Summary

The data indicates that GAMs may not solely inhibit or enhance glioma growth. Rather, the dominant propensity depends on the interacting microenvironment. It is pos-sible that the tumor-supportive role of the microglia is unintentional—a tumor subversion of a physiological response normally used to deescalate immunological reactivity. Typically the CNS microglia are providing a surveillance function of

CNS tissue, guiding the lymphocytes there and exerting their own effector functions and scavenging to wipe out glioma cells. In the early stages of gliomagenesis, innate responses mediated by microglia may be beneficial and involve the activation of effective surveillance by adaptive immunity resulting in the elimination of these cells [136]. However, in the context of progressive malignancy, when tumor cells have escaped immune editing, the smoldering inflammation orchestrated by GAMs, may promote tumor progression. Therapeutic strategies targeting macrophages should take into account the dual role of these cells.

Abbreviations

AchR:	Acetylcholine receptor
ADCC:	Antibody dependent cell mediated cytotoxicity
AIF-1:	Allograft inflammatory factor-1
APC:	Antigen-presenting cell
CpG-ODN:	CpG-oligodeoxynucleotide
CNS:	Central nervous system
DcR3:	Decoy receptor 3
EC:	Endothelial cells
EGF:	Epidermal growth factor
GAMs:	Glioma-associated microglia/macrophages
GSCs:	Glioma cancer stem cells
HGF:	Hepatocyte growth factor
HO-1:	Heme oxygenase-1
HSV-TK:	Herpes simplex virus 1 thymidine kinase
IL:	Interleukin
iNOS:	Inducible nitric oxide synthase
MDSCs:	Myeloid-derived suppressor cells
MHC:	Major histocompatibility complex
MIF:	Macrophage inhibitory factor
MMP:	Matrix metalloproteinase
NO:	Nitric oxide
ROS:	Reactive oxygen species
PACAP:	Pituitary adenylate cyclase-activating polypeptide
PGE2:	Prostaglandin E2
SF:	Scatter factor
STAT:	Signal transducer and activator of transcription
TGF:	Transforming growth factor
TLR:	Toll-like receptor
TMZ:	Temozolomide
TNFR:	Tumor necrosis factor-related
Tregs:	Regulatory T cells
VEGF:	Vascular endothelial growth factor
VIP:	Vasoactive intestinal peptide.

Conflict of Interests

The authors have declared that no conflict of interests exists.

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Review Article

The Leech Nervous System: A Valuable Model to Study the Microglia Involvement in Regenerative Processes

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Microglia are intrinsic components of the central nervous system (CNS). During pathologies in mammals, inflammatory processes implicate the resident microglia and the infiltration of blood cells including macrophages. Functions of microglia appear to be complex as they exhibit both neuroprotective and neurotoxic effects during neuropathological conditions *in vivo* and *in vitro*. The medicinal leech *Hirudo medicinalis* is a well-known model in neurobiology due to its ability to naturally repair its CNS following injury. Considering the low infiltration of blood cells in this process, the leech CNS is studied to specify the activation mechanisms of only resident microglial cells. The microglia recruitment is known to be essential for the usual sprouting of injured axons and does not require any other glial cells. The present review will describe the questions which are addressed to understand the nerve repair. They will discuss the implication of leech factors in the microglial accumulation, the identification of nerve cells producing these molecules, and the study of different microglial subsets. Those questions aim to better understand the mechanisms of microglial cell recruitment and their crosstalk with damaged neurons. The study of this dialog is necessary to elucidate the balance of the inflammation leading to the leech CNS repair.

1. Introduction

Although long underestimated, microglia nowadays comprise an attractive target for accessing the diseased CNS. Microglial cells are regulators of tissue homeostasis in the adult central nervous system and readily participate in pathological processes, orchestrating tissue remodeling. In vertebrates, microglia are currently considered to be a kind of sensor in the brain because they respond to alterations in the brain and are activated by such changes [1]. Microglia constitute the first line of cellular defense mechanisms against central nervous system diseases [2], participating in the regulation of nonspecific inflammation as well as adaptive immune response [3]. That constitutes a very early stage in response to injury [4–6]. When the brain is injured or affected by diseases (e.g., degenerative, infectious, or autoimmune diseases), the resident ramified microglia morphologically transform into cells with retracted processes and enlarged cell bodies and increase in number at the affected site. Microglial cells with this particular form are generally referred to as "activated microglia" or "reactive microglia".

The complexity of microglial responses is reinforced by the cell origin which is still controversial. In addition to the endogenous microglia which result from invasion processes in brain during early embryogenesis, studies showed that myeloid progenitors can penetrate into the brain even in normal adult mice to replace decaying microglial cells. In addition, phagocytes with morphological features of endogenous microglia might be derived from bone-marrow (BM) cells or from circulating monocytes during CNS diseases [7]. The complexity of microglia researches is also increased when we consider that the blood brain barrier is variably broken in these pathologies and that infiltrated cells might subsequently play a critical role in the disease.

Thus the microglial functions appear to be complex as they exhibit both neuroprotective and neurotoxic effects.

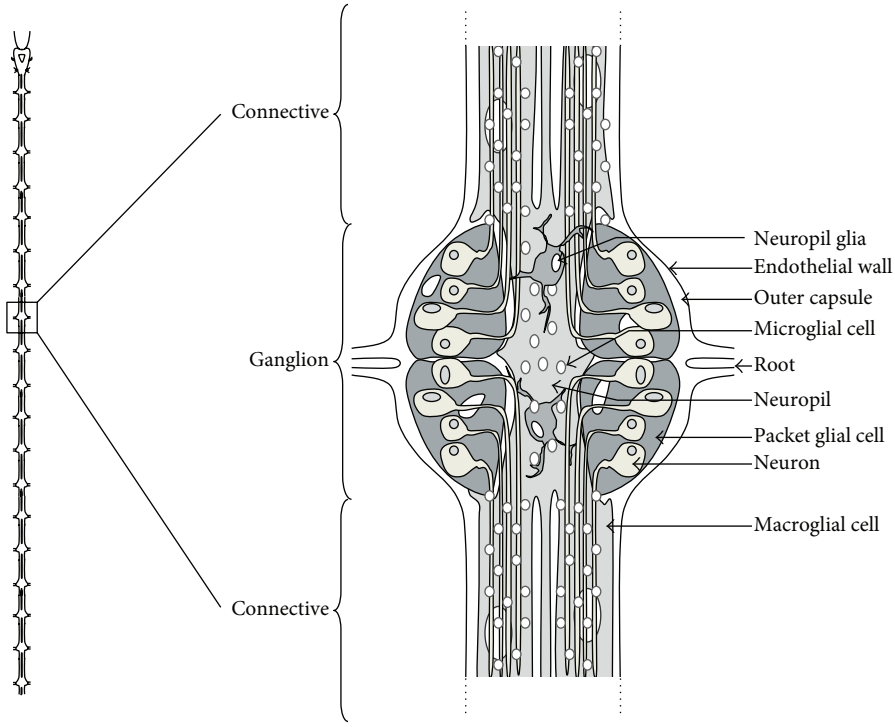


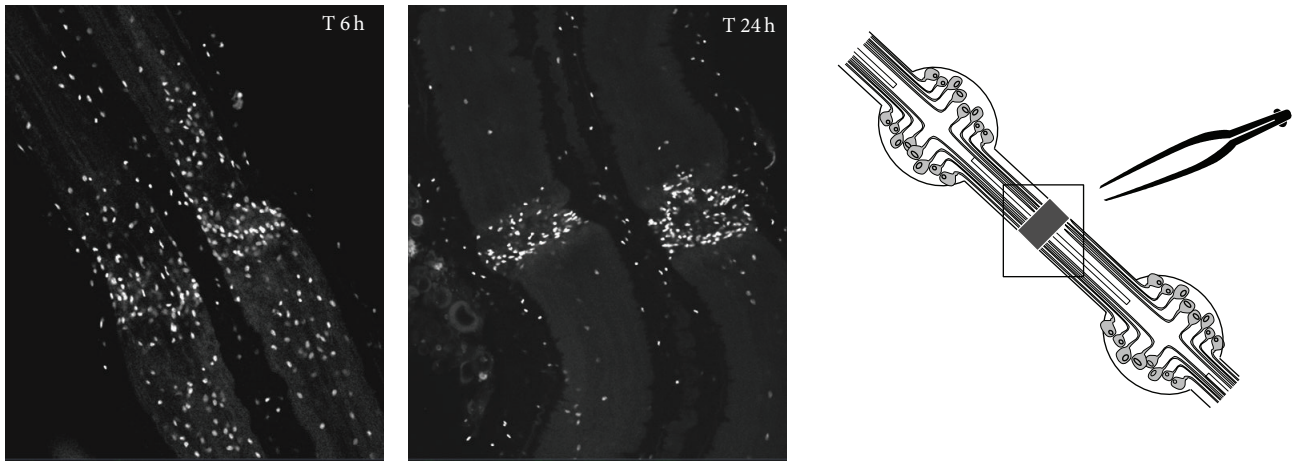
Figure 1: (a) Diagram of leech nervous system containing a head ganglion, 21 body ganglia, and 7 fused tail ganglia joined by connectives. (b) The dorsal view of the ganglion presents packet glial cells enveloping neuron cell bodies. The axonal processes passing through the neuropil are prolonged into connectives. The neuropil lies dorsomedially and contains two macroglial cells. Thousands of microglial cells are distributed in ganglia and connectives. The nervous system is enclosed in the outer capsule which is covered on the outside by a visceral layer of the endothelium (lining the ventral blood sinus). (Reprinted, with permission, from [8]).

In mammals, the CNS is supported and defended by two different macrophage populations: resident microglia and CNS-infiltrating macrophages. Importantly, despite the *in vitro* studies [9], morphological and histological *in vivo* analyses do not allow reliable discrimination of these two cell types because of common changes in form and marker staining. While the importance of "bone marrow-derived microglia" is highly questioned in neurodegenerative diseases, the understanding of the functional differences of infiltrating macrophages is a necessary prerequisite to elucidate successive steps, involving activated microglia, occurring in CNS pathologies [10].

2. The Leech CNS Mapping

Hirudo medicinalis is well studied in neurobiology because the CNS structure is tightly defined for decades [11]. Leech CNS is included in the ventral blood sinus and is constituted by head ganglion, 21 body ganglia, and 7 fused tail ganglia (Figure 1(a)). The ganglia are joined by connectives that consist of two large lateral bundles of nerve fibers and a thin medial connective called Faivre's nerve. Each segmental ganglion contains about 400 neurons and is linked to its neighbors by thousands of axons that form the connectives (Figure 1(b)). The other types of cells in leech ganglia are two connective glial cells that surround the axons, a neuropil giant glial cell and six packet-glial cells that ensheathe the cell bodies of neurons. In the adult CNS, microglial cells are small resident cells evenly distributed in the ganglia (more than 10000 for each one) and in the connectives (2000 for each one).

Since the observations of Retzius in 1891, the nerve cells in leech were studied for their morphological and, later, electrophysiological properties. The simple structure of the nervous system allowed studies about the specificity of synaptic connections. Thus individual neurons were functionally identified and mapped in each ganglion. Firstly, three groups of sensory neurons were discriminated and named touch (T), pressure (P), and nociceptive (N) cells [12]. Secondly, motor neurons (M) were identified [13]. The leech locomotive behavior—the swimming—has been studied following CNS lesions. Several electrophysiological and behavioral analyses allowed the *in vivo* studies of these leech processes. Some authors showed that individual sensory cells develop new synaptic connections with a high degree of specificity after a section, enabling to compensate the effects of the lesion by the restoration of the swimming [14, 15]. Synapse regeneration was demonstrated by *in vitro* experiments from isolated leech ganglia [16]. In addition, another identifiable cell, the single (S) interneuron, facilitated the study of specific connections



(a) (b) (c)

Figure 2: Observation of microglial cell accumulation from isolated and crushed segment of nerve cord maintained in tissue culture (see the diagram (c)). Microglial cell nuclei (white) were stained with Hoechst fluorescent dye to observe cell accumulation at the crush. (a) Six hours following a lesion of connectives, microglial cells progressively migrate toward the lesion site. (b) Later, 24 hours after the crush, microglial cells are mainly recruited to the lesion. This accumulation can be studied as much *in vivo* as *in vitro*.

between its axon and a single target cell. Each ganglion contains one S cell which is connected via its very long axon to that of the other interneuron in the neighboring ganglion. Of interest, unlike the mammalian brain, the leech CNS can be directly manipulated by intracellular injection of protease upon a synaptic target without any damage of the connected axon. In this context, if one S cell's axon is severed, the regenerative processes will form new synapses along its own distal stump leading to a new functional connection with its original target cell [17–19]. The mechanisms of nerve regeneration were progressively investigated by taking into account that other cell types are present close to the injured neurons. One type corresponds to the giant glial cells which are single in each connective and ensheathe axons in this region between adjacent ganglia. Some experiments permitting their individual destruction by intracellular injection of protease showed that, although their absence, the ability of a single damaged axon to reconnect with its specific target is not altered [20–22]. Thus these giant glial cells (macroglia) are not essential to engage the axonal sprouting. Other experiments have focused on another type of glial cells in the leech CNS, the microglia.

3. The Leech Microglia

Although microglial cells were described by a number of authors including Nissl [23], Robertson [24], and Alzheimer [25], they were first named "microglia" by Del Rio-Hortega in the leech CNS using his silver carbonate method [26, 27].

The different studies focusing on the leech microglia were justified by the structure of leech CNS which favors the study of resident microglia activation.

3.1. A First Observation: The Leech Microglia Migrate at the Lesion Site. Most observations of microglia in the leech were made by electron microscopy. Microglial cells have been evoked for the first time for the increase of their number after injury [28]. Morgese and collaborators then used two different histological techniques adapted for leech CNS tissue in order to see microglial cells in whole mount with the light microscope [29]. Weak silver carbonate, a classical stain for vertebrate microglia, allowed them to observe small glial cells similar to their vertebrate counterparts. The authors also used Feulgen's DNA specific staining for determining the distribution of leech microglial cells. Within 24 hours after the nerve cord is crushed, leech microglia aggregate to the site of injury [16]. Interestingly, some experiments were performed on isolated segment of nerve cord maintained in tissue culture (Figure 2). In such preparation, axon and synapse regeneration of the nerve cord has been shown to occur. In isolated connectives that were crushed, microglial cells clustered at the crush. Thus leech microglia can clearly respond to injury in the absence of blood [29]. These authors suggest that, thanks to the appearance of leech microglia following lesions, these cells could be phagocytic. They report an egress of glial cells from traumatized nervous tissue [29]. Observations in the leech suggested that these mobilized microglial cells may be engaged in clearing the damaged tissue of cellular debris. The present paper proposes simple experiments but unrealized on leech microglia to date and brings the evidence of the phagocytic activity of leech microglial cells (Figure 3). The analysis has been realized by using confocal microscopy which allows the observation inside the cells with successive focal plans. Only two focal plans are presented in the figure. The results show that leech microglial cells are able to rapidly phagocyte FITC-labeled bacteria (*Aeromonas hydrophila*). Though bacteria are not yet observed in cells after 1 minute, phagocytosis processes are significantly detectable following 10 minutes of incubation (Figure 3(a)). The mechanism is still observable

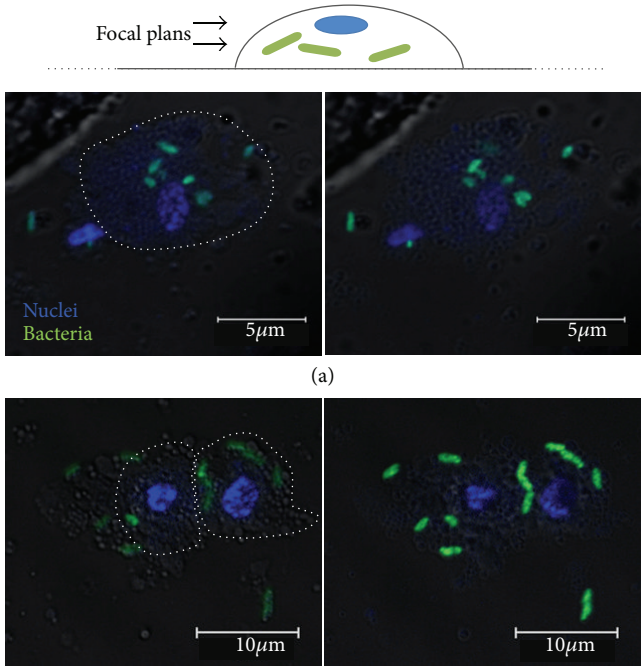


FIGURE 3: Phagocytosis activity of leech microglial cells. Following the dissection of leech nerve cord, microglial cells have been isolated and incubated in Ringer solution. Then heat-killed bacteria (*Aeromonas hydrophila*) have been labeled with fluorescein isothiocyanate (FITC) to be incubated during 1 minute, 10 minutes, 20 minutes, or 6 hours in respective identical plates containing microglial cell culture at room temperature. Finally, after fixation with 4% paraformaldehyde, the cytospinned cells have been incubated with a nuclear dye (10 $\mu g \cdot mL^{-1}$ Hoechst 33342), washed in 0.1 M PBS, and mounted for analysis by confocal fluorescent microscopy (blue, nuclei; green, bacteria). Only 10 minutes (a) and 6 hours (b) conditions are presented in the figure.

after 20 minutes (data not shown) and 6 hours of incubation (Figure 3(b)) although the experiment does not reveal if it involves the same microglial cells or other additional ones in the time course of phagocytosis. That suggests that some microglial cells are able to eliminate debris in a short time. It is interesting to note that some experiments preincubating the cells in 1 mM ATP for 1 hour have been realized in similar conditions. The presence of that known microglial activator did not significantly modify the results in terms of kinetics or phagocyte number.

3.2. During the Response to a Lesion, the Leech Microglial Cells Change Their Morphology. Conformational changes have been described as necessary to allow natural functions of microglial cells [30]. Following a crush in the connectives, microglial cells migrate at the lesion. As previously described in mammalian microglia, this mobility has recently been associated to a transformation from stellate to rounded shape (Figure 4) [31]. The progressive amoeboid feature is comparable to those indicatives of the process of activation in the mammalian immunocytes. Once recruited, the microglial cells interact with damaged neurons allowing neurite outgrowth. Some observations showed that microglial cells need to be ramified to crosstalk with neuritis [32]. Indeed, leech microglial cells can be isolated from a freshly dissected nerve cord. Then microglial cells may be cultured *in vitro*. By using concanavalin-A (Con-A) as a coating, a high percentage of cells are still rounded. When cells are cultured on Con-A in the presence of injured CNS factors, the number of rounded cells decreases to 48% meaning that the other ones are able to develop processes (intermediate form) [30]. This change of morphology allowed more growth of neurites across microglial cells. Thus some diffusible substances from injured CNS tissue are responsible of the morphological transition of the microglial cells. When microglial cells are plated on laminin, they are mainly spindle shaped and interact with growing neurites.

Thus in this context, laminin, a glycoprotein molecule from CNS extracellular matrix, appeared within the connectives in the region of neurite outgrowth suggesting that it may promote axonal growth in the CNS of the animal as in culture [32]. Leech laminin could provide a favourable substrate that induces efficient neurite outgrowth of some neurons in culture, but the cells which produce laminin at the crush site have to be identified. Even following the ablation of giant glial cells, microglial cells were concentrated at the sites of new laminin appearance and axon sprouting. This observation suggests that microglial cells may be responsible for the appearance of new laminin molecules contributing to sprouting [30, 33].

As a result, diffusible factors including laminin could be necessary to condition the functional features of microglia in their crosstalk with damaged neurons at the lesion site.

3.3. Dynamical Studies of Microglia Recruitment: Structural Advantages. The previous data show that leech microglial cells have similar properties with mammalian counterparts through their mobility, their phagocytic activity, and their morphological changes during activation processes. Interesting features of the leech nerve cord structure can explain the story of the leech neurobiology. Because the central nervous system (CNS) is organized as a tubular nerve cord with adjacent ganglia joined by connectives, it facilitates the study of the nerve cells. The neuronal cell bodies are mapped in each ganglion [34] processing their axon into the connectives. It firstly results in that mechanisms following nervous system injury can be analyzed at the single cell level [19]. Since the microglia accumulation at the lesion site is considered as an important and rapid recruitment to engage axonal sprouting, it secondly results in that this cell movement can be dynamically studied by *in vivo* imaging of the connectives. Importantly, microglia cannot be confused with other cells because there are neither astrocytes nor oligodendrocytes in leech CNS. Finally, the absence of blood vessels within the central nervous system does structurally simplify the separation between CNS and blood. Thus the leech inflammatory response may be explored from the sinus-free CNS. Anyway, the very low infiltration of blood cells which have been observed in injured CNS—even surrounded

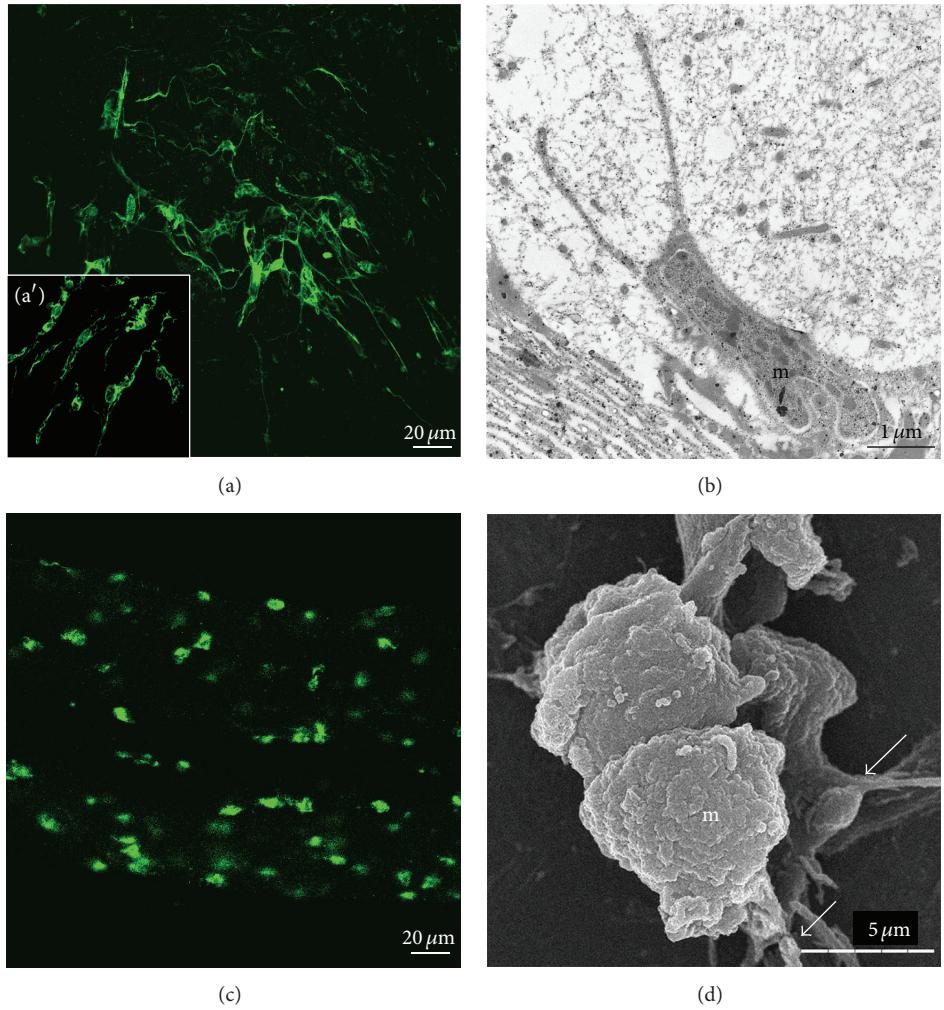


Figure 4: Transformation of resident microglia (m) into activated cells allowing their migration to the injury site. (a) and (c) Immunohistochemical staining using anti-gliarin antibody in ramified microglial cells from connectives (a), ramified microglial cells from ganglia (a′), and rounded microglial cells during their recruitment in connectives (c). (b) Transmission electron microscopy of an elongated microglial cell (m) with thin pseudopods in a naive ganglion. (d) Scanning electron microscopy of activated microglial cells accumulated at the lesions. Once recruited, the cells do progressively develop new ramifications (arrows). (Reprinted, with permission, from [31]).

by the blood sinus—highlights the importance of the resident microglia at the lesions [29, 35]. As a result, leech microglial cells may be studied for their contribution to the CNS repair without any infiltrating blood cell contribution. Since video microscopy is possible to observe Hoechst-stained microglial cells moving to the lesion site, some studies—using isolated segment of nerve cord maintained in tissue culture—showed the migration kinetics of these cells into the leech connectives [36]. Thus, microglia start to move within the first minutes after the injury and accumulate at the lesion site within 2 hours. During this recruitment, only a fraction (less than 50%) is able to move at any time at speeds up to 7 μm per minute [36].

Because the microglia accumulation at the site of lesion is known as a rapid and important process for the usual sprouting of injured axons in leech *Hirudo medicinalis*, the subsequent studies aimed to identify the molecular processes responsible of the microglial recruitment following lesions in the leech CNS.

3.4. Molecular Mechanisms Involved in Microglial Migration

3.4.1. Nitric Oxide and Purines. A first explanation of molecular mechanisms came from the endothelial nitric oxide synthase (eNOS) activity in the injury site as a very early response suggesting that NO is involved in the microglial recruitment at the lesions [37]. Moreover, some experiments using NO inhibitors resulted in a decrease of microglial cell accumulation suggesting that NO contributes to microglia chemotaxis [38]. Importantly, the use of spermine NONOate (SPNO) as an NO donor at the lesions also reversibly blocked the accumulation of microglia [38]. These data suggest that NO could attract the microglial cells at low concentrations

(distant from the lesion site) promoting their movement and also serves as a stop signal for migrating microglia at a high concentration (at the crush). Further studies using NO microsensor demonstrated the rapid efflux of NO at the lesions controlling the microglial accumulation [39]. Nitric oxide is also known to activate a soluble guanylate cyclase (sGC) leading to cyclic GMP (cGMP) production. Immunohistochemical experiments using anti-eNOS and anti-cGMP antibodies showed a similar increase of eNOS and cGMP at the lesion site. When methylene blue is used as a soluble guanylate cyclase (sGC) inhibitor, the cGMP immunoreactivity is abolished which is correlated with the inhibition the microglial accumulation at the lesion [40]. Actually, the decrease of cell number at the lesion results from the misdirection of moving cells. The use of NO scavenger (cPTIO) confirmed the involvement of NO in a cGMP-dependent signaling pathway by decreasing the cGMP immunoreactivity [41]. Importantly, the NO scavenger did not reduce the microglial cells movement but altered their directionality. In mammals, NO/cGMP signaling pathway activates a protein kinase G, called PRKG1 (alias cGK1 or cGKI), which phosphorylates serines and threonines on many proteins leading to the modulation of cellular calcium. Proteins that are phosphorylated by PRKG1 are then involved in the CNS to promote, for example, the axon guidance [42]. Although the functional involvement of PRKG1-related molecule has not been so far specified in NO-dependent activities in leech CNS, we have recently identified at least two molecules presenting high similarities with mammalian and invertebrate PRKG1 homologs. Further studies would focus on their cell localization in order to better understand NO effects on target cells. Thus, NO is the first diffusible molecule identified in the leech CNS that is essential to organize the microglial movement toward the lesion. Other molecules are able to engage the leech microglial movement. Indeed ATP, ADP, and UTP have been investigated for their ability to activate the microglial accumulation [41, 43]. Though ATP could be released via innexins from glia to activate microglial cells [44, 45], further studies are still necessary to identify the leech receptors for such chemotactic molecules. The use of reactive blue 2 (RB2) as an antagonist of purinergic receptors slowed the migration (but did not misdirect the cells in movement) suggesting the existence of such a receptor [41]. The analysis of the leech genome since these last two years hypothesizes the presence of purinergic receptors in leech CNS but has to specify their functional features (unpublished data).

By taking into consideration the advantage of the crushing on an isolated segment of nerve cord, Ngu and colleagues [43] used specific inhibitors for ATP- or NO-dependent activities in order to reduce the microglial cell accumulation (by slowing or disorientating the cells) and then study the consequences on the growth of severed axons. To measure this correlation, sensory neurons were injected with biocytin to label their sprouting after lesioning. Remarkably, in spite of the lesion, when microglial accumulation has been reduced by inhibitors, the authors observed a significant reduction in total sprout lengths on damaged neurons. Consequently, microglia are shown to be essential for the usual sprouting of injured axons [43].

ATP and NO are diffusible molecules presumably released within the first minutes following CNS lesions. Because we know that the microglial accumulation is a rapid but progressive process, other studies have been directed to identify the released factors which are chemotactic for microglial cells in the time course of the accumulation.

In order to characterize potential chemotactic factors involved in the recruitment of the microglial cell population, some studies have taken advantage of the establishment of databases such as Expressed Sequence Tag (EST) library from the leech CNS and the leech genome [46]. According to the sequence homologies with known chemotactic factors and the presence of conserved domains, some products have been functionally investigated.

3.4.2. HmEMAPII. A molecule homologous to the human complex p43/endothelial monocyte-activating polypeptide II (EMAPII) was recently characterized in the leech CNS [47]. In mammals, the p43 precursor is known to be released after processing the cytokine EMAPII which is suggested to be a marker of microglial cell reactivity [48–50]. EMAPII is highly produced in activated microglia of injured brain suggesting its involvement in inflammatory and neurodegenerative pathologies [51, 52].

Human EMAPII has been shown to exert a chemoattractant effect on both leech and human microglial cells. Although it is considered important in microglial activation, its chemotactic function has been described for the first time in the leech microglial [47]. The leech gene coding for *Hm*EMAPII (for *H. medicinalis*) results from a signaling pathway associated with a recently characterized leech TLR in the CNS, called *Hm*TLR1 [47, 53]. Since the CXCR3 molecule is the natural receptor for human EMAPII, chemotaxis assays on leech microglia have been performed using either anti-CXCR3 antibody or IP-10, an agonist of CXCR3. The results showed a competitive effect allowing the reduction of EMAPII-induced microglia migration, suggesting the existence of a CXCR3-related molecule in the leech [47].

3.4.3. HmIL-16. The importance of interleukin-16(IL-16)-related molecule has been described in the microglia recruitment of the leech CNS [31]. In mammals, IL-16 is a proinflammatory cytokine originally identified as a lymphocyte chemoattractant factor [54, 55] and produced by numerous cells including lymphocytes and microglia [56]. The mature IL-16 corresponds to C-terminal peptide of a precursor which subsequently acts by forming bioactive multimers [57]. Indeed, the homotetramerization of human IL-16 was reported as the optimal biological activity [58]. In human brain, IL-16 is constitutively expressed by a microglia subpopulation and may attract CD4+ lymphocytes across the blood brain barrier under pathological conditions [59]. A paracrine role of IL-16 was also demonstrated in inflammation following cerebral ischemia [60]. IL-16 may be implicated in some neurodegenerative diseases such as multiple sclerosis (MS) where enhancement of IL-16 production suggests a role in the regulation of inflammation in axonal damages [60–63].

In the leech, a new molecule, designated *Hm*IL-16, is produced in naïve neurons but rapidly induced following a lesion and transported along the axonal processes to promote the recruitment of microglial cells to the injured axons [31]. *Hm*IL-16 can be produced in microglia at least 72 hours after the injury. Thus microglial cells might first be activated by neuronal *Hm*IL-16 which is released from damaged neurons. Then these recruited microglial cells could release their own cytokine to maintain cell accumulation at the lesion. *Hm*IL16 possesses functional homologies with its human counterpart, by exerting chemotactic activity as it has been similarly observed using human IL-16 on leech microglial cells. By using *in vitro* chemotaxis assay, preincubation of microglial cells either with an anti-human IL-16 antibody or with anti-*Hm*IL-16 antibody significantly reduced microglia migration induced by injured leech CNS-conditioned medium. Remarkably, functional similarities have been demonstrated by the ability of *Hm*IL-16 to promote human CD4+ T cell migration [31].

The fact that *Hm*IL-16 has no effect on CD8+ cell migration further indicates a similar relationship with CD4 as described for human IL-16. Interestingly, the activity of *Hm*IL-16 contained in the conditioned medium has been inhibited by using a soluble CD4 as competitor for the membrane CD4. Although a CD4-related protein has not been identified in leech to date, these results would strongly suggest its presence. Indeed, these results indicate that *Hm*IL-16 might act via a CD4-related molecule on the leech microglial cells as reported for human microglia [59]. Preliminary results using the human anti-CD4 antibody allowed the reduction of human IL-16-induced leech microglia migration (unpublished data). Finally, the same antibody evidenced the presence of an immune-reactive product in leech CNS and in leech protein extracts eluted on affinity column using IL-16-coated beads (unpublished data). The following study is still in progress and would presumably lead to the characterization of such a related molecule in the leech.

3.4.4. HmC1q. A molecule homologous to vertebrate C1q has been recently characterized in the leech CNS [8]. In vertebrates, members of C1q family, C1qTNF proteins are involved in triggering and regulation of various inflammatory reactions [64]. Among the mediators expressed by microglial cells and neurons, C1q seems to be a key molecule in neuroinflammatory diseases, that is, systemic lupus erythematosus (SLE) [65, 66]. It is also involved in various neurodegenerative pathologies as Alzheimer disease [67, 68]. In addition, C1q is known to drive microglial activation [69]. Beside the nervous system, the chemotactic properties of C1q have been demonstrated for immune cells [70–72]. Finally, their migration is mediated through recognition of both gC1qR and cC1qR [73].

In the leech, *Hm*C1q is produced at least in neurons and glial cells. In chemotaxis assays, leech microglial cells were firstly demonstrated to respond to recombinant human C1q [8]. Then, while the use of injured leech CNS-conditioned medium, as *in vitro* chemoattractant, exhibited a significant dose-dependent chemotaxis on leech microglia, its effect was reduced when cells were incubated with anti-*Hm*C1q antibodies. Thus, *Hm*C1q released in the culture medium contributes through its chemotactic effect to the microglial recruitment. Its activity has been correlated to nitric oxide since the NO scavenger (cPTIO) partially abolished the *Hm*C1q effect on the leech microglia recruitment. This result highlights that the C1q effect is related to the presence of NO which plays a key role in leech microglia recruitment as discussed above. *Hm*C1q activity were reduced when microglia were preincubated with signaling pathway inhibitors such as pertussis toxin or wortmannin suggesting the potential involvement of G-proteins and phosphoinositide 3-kinases in the pathway of the C1q-induced signal for chemotaxis [8]. Remarkably, again in chemotaxis assays, when the cells have been preincubated with anti-human gC1qR antibody, the *Hm*C1q effect has been significantly reduced. That result suggests the involvement of gC1qR-related molecule in C1q-mediated migration in leech.

The production of the recombinant form of *Hm*C1q permitted specifying its importance in the microglia migration into the connectives [74]. As discussed above, *ex vivo* experiments can be performed on the isolated segment of nerve cord maintained in tissue culture. In such preparation, it is interesting to note that microglial cells are still reactive and recruited in crushed connectives. This original feature allows the injection of chemotactic factor with or without inhibitors to enhance and/or regulate the Hoechst-dyed cell accumulation. By taking advantage of this natural ability, recombinant *Hm*C1q exhibited a strong chemotactic effect on microglial cells which has been abolished when recombinant *Hm*C1q is simultaneously injected with anti-*Hm*C1q antibody (Figure 5).

Then, the existence of a receptor for *Hm*C1q (called *Hm*gC1qR or *Hm*C1qBP) in the leech CNS has been demonstrated following its molecular characterization from leech databases [74]. The involvement of *Hm*C1qBP in *Hm*C1q-dependent chemotaxis has been definitely ascertained showing the inhibition of *Hm*C1q effect on *in vitro* leech microglia migration by using anti-C1qBP antibodies. Finally, affinity purification and flow cytometry experiments showed the interaction between *Hm*C1q and *Hm*C1qBP molecules. In mammals, such interaction was identified in dendritic cells but has never been shown in nerve cells [73]. In addition, the interaction between *Hm*C1q and its receptor is the first evidence of the molecular reactivity of microglial cells during their accumulation. Interestingly, that receptor has been localized only in a portion of microglial cells (Figure 6). Thus, a well-defined subpopulation of Hoechst-dyed microglial cells recruited at the crush is *Hm*C1qBP positive and reactive to *Hm*C1q demonstrating that microglia cannot be considered as a whole reactive population.

We presently know the involvement of different chemotactic factors in the microglia recruitment to the lesion. Additional studies might evaluate the activity of their respective recombinant form in a time course and a dose-dependent manner on microglia recruitment. That question is crucial to better understand the hierarchy of chemoattractants and the chronological responses delivered to damaged neurons.

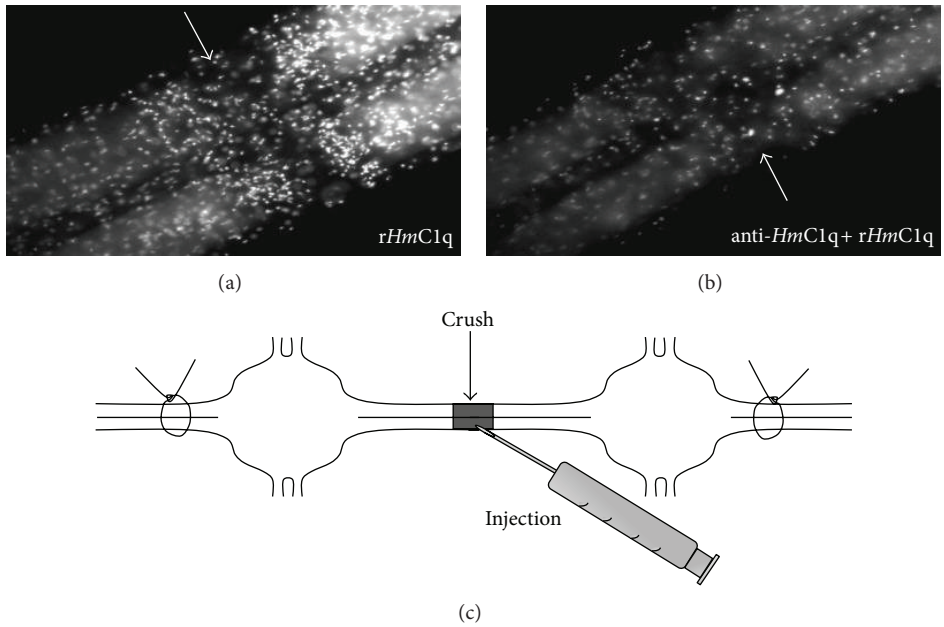


Figure 5: Chemotactic effect of recombinant *Hm*C1q on leech microglia in isolated and crushed segment of nerve cord maintained in tissue culture (see the diagram (c)). (a) Hoechst-dyed microglial cells strongly accumulated 4 hours after injection of r*Hm*C1q (arrows). (b) When tissues have been injected with polyclonal anti-*Hm*C1q antibody, the chemotactic activity of r*Hm*C1q has not been observed (arrows). To note, the corresponding preimmune serum did not exert any neutralizing effect (data not shown). (Reprinted, with permission, from [74]).

It is also necessary to continue the identification of the molecules involved in microglial activation to fully understand their functional relevance. A recent study highlighted the importance of endocannabinoids, namely, N-arachidonylethanolamide (AEA) and 2-arachidonyl glycerol (2-AG), in the balance of NO and ATP release contributing to the microglia accumulation at the crush [75]. In addition to chemotactic factors, the identification of molecules regulating the balance of inflammatory response and activating the microglia/neuron crosstalk would help to understand the microglial functions in the leech CNS repair.

4. Role of Activated Microglia and Crosstalk with Damaged Neurons

The existence of different microglial subpopulations depending on their activating factors is strongly suggested in the leech CNS. Firstly, microglial cells have to be discriminated as a reactive population and nonreactive population. Indeed, injured leech CNS-conditioned medium is able to attract only a part of a whole microglia population.

The involvement of several activating and migrating signals acting on different subsets of microglial cells at the lesion site could be taken into account as suggested for mammals [10, 76]. The leech receptors for every chemoattractant of interest will allow the discrimination of microglial cell population depending on their reactivity to respective chemoattractant. Beside the existence of different activation molecules, it is necessary to understand the functions of recruited microglial cells at the end of damaged axons. Because microglial cells can be isolated from injured CNS, they are maintained in culture in order to be stimulated by respective chemotactic factors (C1q, EMAPII, or IL-16). The molecules from activated microglia which are secreted in the medium can be identified by using potent proteomic tools (nano-liquid chromatography coupled to orbitrap MS analysis) and by analyzing the leech genome. When compared to each other and with unstimulated microglia secretome, the analyses of differential secretomes already revealed products of interest depending on the activation processes (unpublished data). The identification of such products would help to understand the functions of only resident microglia at the lesion. Indeed, unlike mammals, the microglial accumulation in injured leech CNS does not involve any infiltrated blood cells. In mammals, it is not easy to discriminate the role of infiltrating macrophages sharing the same molecular markers as the resident microglia. Moreover, more and more studies show that infiltrated macrophages can exert distinct functions in the balance of inflammation depending on their polarization between M1 (pro-inflammatory) and M2 (neuroprotective) phenotypes [77]. Because nerve repair spontaneously occurs in leech nerve cord, we can hypothesize the existence of a particular neuroprotective microglial phenotype. In order to achieve the functional properties of activated microglial cells at the lesion, each secretome would be tested for its capacity to outgrow damaged neurons *in vitro*. By taking into consideration the advantage of the leech CNS facilities, notably the *in vivo* study of the axonal sprouting [16, 17], the injection of regenerative secretomes directly on crushed connectives would be possible in order to measure the time course of regenerative processes. Actually, some

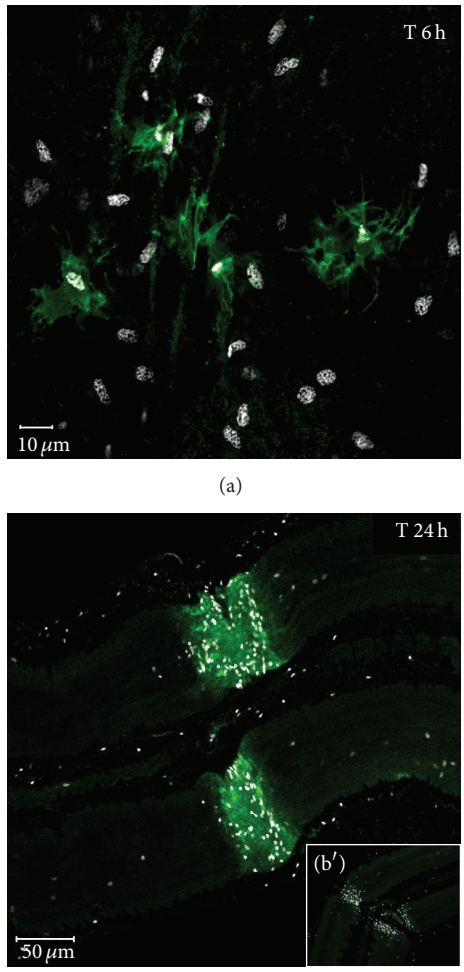


Figure 6: Immunostaining of leech central nervous system (CNS) using rabbit polyclonal anti-human C1qBP antibodies (green). Microglial cell nuclei (white) were stained with Hoechst fluorescent dye to observe cell migration. (a) High magnification image of the injury site after 6 hours. Since all microglia nuclei are shown by Hoechst counterstaining (white), images demonstrate that the anti-C1qBP immunostaining selectively enhances some microglial cells. (b) 24 hours following injury, the number of microglial cells is much higher at the lesion site, and stronger positive immunostaining is observed. (b′) No immunostaining was observed using secondary antibodies alone as negative controls. (Reprinted, with permission, from [74]).

links might exist between the leech neuroinflammation and mammalian degenerative diseases since we observed that leech molecules interact with human immune cells [31] and that human factors act on leech microglia [8, 31, 47]. The different factors which were described in the leech CNS are more and more studied in mammals in neuroinflammatory processes as, for example, the human C1q which is a microglia activator [69]. These elements led us to investigate a possible link between the fundamental research and clinical application by using leech molecules in the rat spinal cord injury (SCI) model. The potential neuroprotective roles of leech CNS-secreted molecules have been assessed on rat SCI model in collaboration with Pr. Cizkova (Slovak Academy of Sciences, Kosice, Slovakia). Preliminary results using leech CNS-conditioned medium on dorsal root ganglion (DRG) neurons from adult rat show a rapid and significant neurite outgrowth (unpublished data).

5. Conclusion

Unlike vertebrates, some invertebrate animals can fully repair their central nervous system (CNS) following an injury. The CNS of medicinal leech is efficiently and functionally regenerated following lesions [14, 15]. In this context, the implication of microglia is a key step to engage an adapted response leading to the axonal sprouting. To summarize, in contrast to mammalian CNS, distinctions between the role of resident microglia and that of blood infiltrating macrophages—which contribute to neuroinflammatory mechanisms in mammals—are possible in leech CNS. Considering its easy manipulation and structural context, the leech CNS allows the *in vivo* and *in vitro* studies of activated microglial cells. This valuable model would offer interesting molecular and cellular bases to evaluate the consequences of microglia involvement in regenerative processes.

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Review Article

Microglia and Macrophages in Malignant Gliomas: Recent Discoveries and Implications for Promising Therapies

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Malignant gliomas are the most common primary brain tumors. Their deadliest manifestation, glioblastoma multiforme (GBM), accounts for 15% of all primary brain tumors and is associated with a median survival of only 15 months even after multimodal therapy. There is substantial presence of microglia and macrophages within and surrounding brain tumors. These immune cells acquire an alternatively activated phenotype with potent tumor-tropic functions that contribute to glioma growth and invasion. In this review, we briefly summarize recent data that has been reported on the interaction of microglia/macrophages with brain tumors and discuss potential application of these findings to the development of future antiglioma therapies.

1. Introduction

Malignant gliomas, the most common primary brain tumors that arise from glial cells within the central nervous system (CNS), are among the most fatal human cancers [1]. Glioblastoma multiforme (GBM), the most aggressive type of malignant glioma, is highly invasive, making tumor recurrence certain even after a complete resection [2]. Besides, the presence of the blood-brain barrier (BBB) significantly limits the penetration of most chemotherapeutic agents into the CNS [3]. With a median survival of only 14.6 months even after aggressive therapy with surgery, radiation, and chemotherapy, most patients succumb to their disease within two years of the initial diagnosis [4]. Thus, there is a pressing need for discovery of more effective therapies to improve patient outcomes.

Malignant gliomas are heavily infiltrated by myeloid-derived cells (recently reviewed by Kushchayev et al. [5]). Among these, tumor microglia and macrophages appear to be the most common cells in brain tumors. Tumor microglia arise from resident CNS macrophages, while circulating monocytes give rise to glioma-associated macrophages. In experimental glioma models, tumor microglia and macrophages can be differentiated by FACS based on CD45 and CD11b staining characteristics [6], but in human tissue samples, such separation is not as distinct. Although both cell types can acquire M1 phenotype and are capable of releasing proinflammatory cytokines, phagocytosis, and antigen presentation [7], their effector immune function in gliomas appears to be suppressed. In fact, increasing new evidence suggests that microglia and macrophages interact with the tumor cells by promoting their growth and migration [8]. In this review, we briefly summarize recent data that has been reported on microglia/macrophages brain tumor interaction and discuss potential application of these findings to the development of future antiglioma therapies.

2. Chemoattraction

Glioma-associated microglia and macrophage (collectively referred to as GAMs here) compose approximately 30% of

tumor inflammatory cells and are actively recruited by gliomas through secretion of a variety of factors including chemokines, cytokines, and matrix proteins [9–13]. Among chemokine pathways involved in TAM chemoattraction, CCL2 (monocyte chemotactic protein-1 (MCP-1)) was among the first identified in gliomas [14]. Although CCL2 expression can be induced by a variety of stimuli and cytokines, mechanisms responsible for its baseline expression by gliomas are being studied. Adenosine-5′-triphosphate (ATP), for example, was shown to stimulate the production of chemokines MCP-1 and interleukin-8 (IL-8) in gliomas [15]. Recently, we demonstrated that in a subgroup of gliomas, protein S100 calcium binding protein B (S100B) may also play a role in MCP-1 upregulation and GAM recruitment [16]. A direct correlation between the percentage of GAMs and MCP-3 expression levels has also been demonstrated in human gliomas, suggesting MCP-3 to also participate in microglia/macrophages chemoattraction [12].

Stromal-derived (SDF-1) factor-1 is another chemokine that has been shown to promote microglia/macrophage trafficking in gliomas [17]. Trying to recapitulate neuropathological features of human high-grade glioma, Wang et al. established a new murine brain tumor model, ALTS1C1, which expresses high levels of SDF-1. To unveil the role of SDF-1 in this tumor model, the expression of this chemokine in tumor cells was inhibited. The density of microglia/macrophages in the SDF-knockdown tumor was higher in nonhypoxic than in hypoxic regions, suggesting that SDF-1 production by tumor cells might be crucial for the accumulation of microglia/macrophages into areas of hypoxia and tumor invasiveness [13].

Glioma and GAMs participate in a number of paracrine networks that promote their coexistence. Glioma cells constitutively express colony stimulating factor-1 (CSF-1) that stimulates microglia invasion through its receptor CSF-1R. Synergistically, microglia stimulate glioma cell invasion through epidermal growth factor receptor (EGFR) activation [10]. Further, in response to glioma cells, microglia express tumor necrosis factor receptor of mouse embryo (TROY) that drives microglia migration towards glioma cells [18]. Also, the chemokine CX3CL1 expressed in glioblastoma cells promotes recruitment of human microglia/macrophages through its receptor CX3CR1 and enhances the expression of matrix metalloproteases 2, 9, and 14 in these cells, possibly promoting tumor invasion [11].

Glioma-initiating and cancer stem cells also have a role in recruiting microglia/macrophages. The former promote microglia migration through chemokines CCL5, vascular endothelial growth factor (VEGF) and neurotensin (NTS) release [19], while conditioned medium from the latter was shown to induce the migration of human monocytes [20].

3. Immunosuppression

After attracting microglia/macrophages, tumor cells establish an immunosuppressed microenvironment, leading GAMs to acquire an alternatively activated (M2) phenotype that further contributes to the local immunosuppression and supports tumor growth and invasion [8, 21, 22]. Recently, we demonstrated that S100B, a protein that is expressed by most gliomas and activates receptor for advanced glycation end products (RAGE) on microglia/macrophages, can induce signal transducer and activator of transcription 3 (STAT3) activity, resulting in suppression of microglia and primary monocyte function *in vitro*, reflected by inhibition of interleukin-1 beta (IL-1β), tumor necrosis factor-alpha (TNF-α) production, and other pro-inflammatory cytokines [23]. As a signal transducer, STAT3 is a central node for numerous oncogenic signaling pathways involving cytokines and growth factors [24]. STAT3 is also an important transcription regulator, defining a transcriptional program at multiple levels that facilitate tumor cell proliferation, survival, invasion, cancer-promoting inflammation, and suppression of antitumor immune responses [24].

GAMs have also been shown to express a variety of immunosuppressive cytokines. For example, transforming growth factor-beta 1 (TGF-β1), an immunosuppressive cytokine that is expressed by glioma cells, is also produced by GAMs [25]. This process may be in part mediated by release of macrophage inhibitory cytokine-1 (MIC-1) by glioma stem cells [20].

4. Promotion of Tumor Growth and Invasion

Wesolowska et al. have shown that TGF-β1 originated from microglia is crucial for promotion of glioma invasion. Interestingly, primary microglia cells cocultured with glioma cells drastically increased the secretion of TGF-β1 [26]. More recently, another research group confirmed that the TGF-β1 released by microglia/macrophages enhances the invasive capacity of the $CD133^+$ glioma cells (glioma stem-like cells) more than the $CD133^-$ committed cells, and that this process most likely occurred through type II TGF-β receptor. Moreover, this invasion was suggested to be promoted by an increase of MMP-9 (matrix metalloprotease 9) expression in the $CD133^+$ glioma cells [27].

Metalloproteases play an important role in microglia/macrophage-mediated glioma migration. Conditioned medium from microglia cells incubated with S-Adenosylhomocysteine (SAH) promotes the invasion of a glioma cell line in a SAH-dose-dependent manner. This occurs through the increase of MMP-2 expression and activity and the decrease of the tissue inhibitor of metalloprotease-2 (TIMP-2) in microglia-treated cells [28]. Markovic and colleagues have elegantly shown that membrane type I metalloprotease (MT1-MMP) is upregulated in GAMs and that the tumor itself induces the expression and activity of MT1-MMP [29]. This microglial metalloprotease in turn seems to activate MMP-2 in gliomas, leading to even higher tumor invasiveness [29]. GAMs also express CX3CR1 and, in response to CX3CL1, increase their adhesion, migration, and expression of MMP-2, -9, and -14 [11].

Cytokines and chemokines are also important in glioma growth and invasion. Overexpression of CCL2 in the U87 glioma cell line was recently shown to enhance its invasiveness in a three-dimensional collagen matrix when these

cells were cocultured with microglia (which express the CCL2 receptor CCR2) [30]. Furthermore, levels of IL-6 were increased in the coculture medium, and the expression of IL-6 *in situ* corresponded to the microglia/macrophages. Also, incubation of the glioma cells with recombinant IL-6 significantly increased their invasion, a process that was reversed with an IL-6 neutralizing antibody. These findings suggest that gliomas exploit microglia/macrophages through a CCL2/CCR2/IL-6 loop to increase their invasion and migration [30]. Intense angiogenesis is another hallmark of malignant gliomas whereby these tumors obtain essential nutrients and oxygen [31]. Loss of Flt-1, a VEGF receptor, signaling in microglia/macrophages leads to a decrease in tumor growth and vessel density, confirming an active role of microglia/macrophages in glioma angiogenesis [32].

Recently, it was demonstrated that the cochaperone stress inducible protein-1 (STI1), a cellular prion protein (PrP^c) ligand, released by primary microglia cells promotes proliferation and migration of glioma cell lines in a PrP^c-independent fashion (possibly involving MMP-9). Moreover, this proliferative effect was specific to brain macrophages, since conditioned medium from peritoneal macrophages was unable to induce glioma cells growth [33]. As previously discussed, glioma and microglia interactions mediated through EGF and CSF-1 can also increase tumor invasion [10].

5. Promising New Therapies

As detailed in this and other reviews, microglia/macrophages are recruited to the glioma site, but their immune effector function is impaired, and these cells can even promote tumor growth and invasion. Attempts are being made to identify new targets that can reverse this GAM function. One potential candidate is STAT3. Our group showed that conditioned medium from glioma cells increased STAT3 activity in microglia cells, resulting in overexpression of IL-6 and IL-10 and downregulation of IL-1β [34]. When STAT3 was inhibited *in vitro* by a pharmacological agent or small interfering RNA (siRNA), the expression profile of these cytokines was reversed. Moreover, silencing of STAT3 in a murine glioma model resulted in microglia/macrophages activation and tumor growth inhibition [34]. Likewise, corosolic and oleanolic acids have been shown to suppress STAT3 in macrophages [35, 36]. These compounds also inhibited the expression of CD163, a marker of the M2 phenotype and the secretion of IL-10 in human macrophages, suggesting that they could potentially be used to suppress the M2 polarization of microglia/macrophages [35, 36].

The use of antibodies to alter GAM function also has been under investigation. Systemic administration of neutralizing antibody against CCL2 significantly inhibits the infiltration of microglia/macrophages in mice bearing gliomas [37]. Furthermore, the combination of anti-CCL2 therapy with chemotherapy (temozolomide) significantly prolonged the survival of mice, suggesting a possible new therapeutic approach [37]. Furthermore, antiphosphatidylserine antibody combined with irradiation was also investigated in a rat model of glioblastoma. Antiphosphatidylserine binds to tumor vascular cells exposing phosphatidylserine in response to irradiation and induces antibody-dependent cell-mediated cytotoxicity by CD11b-positive macrophages. This resulted in the death of tumor cells through starvation and significantly increased the median survival time of tumor-bearing animals [38].

Taking advantage of their phagocytic properties, our group is actively pursuing the development of nanoparticles for modulation of microglia/macrophage activity in brain tumors. Carbon nanotubes (CNTs) and cyclodextrin-based polymer are semiselectively taken up by GAMs with no toxicity [39, 40]. These nanoparticles can be used to enhance the uptake of CpG oligonucleotides, an agonist of toll-like receptor 9 (TLR-9), by glioma-associated inflammatory cells. This strategy was used to overcome the local tumor immunosuppression and eradicated gliomas in mice models [41].

Moreover, Kopatz et al. used another approach to boost the phagocytosis property of microglia cells in a glioma model. Microglia were stimulated to express sialic-acid-binding immunoglobulin-like lectin-h (Siglec-h) that bound to intact glioma cells, but not normal brain cells, resulting in the engulfment of tumor cells, a process dependent on DAP12, an adapter molecule, signaling [42].

Microglia/macrophages may also function as delivery vehicle into brain tumors [43]. Baek and colleagues reported using murine macrophages as carriers of nanoshells (NS) into gliomas for photothermal ablation. NS composition efficiently converts absorbed near-infrared light (NIR) into heat and exposure of glioma spheroids infiltrated with NS-loaded macrophages to NIR laser resulted in complete tumor growth inhibition [44].

Other well-known agents can also be used as antiglioma therapies by targeting GAMs. The antibiotic minocycline hydrochloride is a small, highly lipophilic molecule that is readily absorbed after oral administration and capable of crossing the BBB. Markovic et al. have shown that minocycline attenuates the protumorigenic effect of GAMs by inhibiting p38 MAP kinase that is responsible for MT1-MMP upregulation in microglia [45]. Besides, the inhibition of p38 MAPK was also shown to reduce the secretion of pro-inflammatory cytokines from microglia and tumor cells, resulting in a decrease of glioma migration [46]. Moreover, systemic administration of cyclosporine A (CsA) was shown to decrease glioma growth and angiogenesis by inhibiting microglia/macrophages infiltration by inducing cell death and blocking the expression and activity of important enzymes and cytokines required for tumor invasion [47]. Propentofylline (PPF), an atypical methylxanthine and glial modulating agent with anti-inflammatory actions, was also shown to be effective in reducing glioma growth by targeting microglia and possibly decreasing their expression of MMP-9 [48].

Other immunotherapy approaches that target GAMs include stimulation of microglia by IL-12 to increase their phagocytic activity and TNF-related apoptosis inducing ligand (TRAIL) release [49]. Also, stimulation of human GAMs by the TLR3 agonist poly(I:C) results in tumor cell death and inhibition of tumor cell growth and invasion [50]. Finally,

because microglia/macrophages express folate receptor β (FRβ), a recombinant immunotoxin to FRβ has been used to deplete GAMs in order to decrease tumor growth [51].

Integrins can also be targeted to attenuate glioma growth. In an organotypic brain culture containing glioma cells, the inhibition of alpha5 beta1 ($\alpha5\beta1$) integrin resulted in a significant decrease in tumor growth. Interestingly, depletion of microglia cells abolished the effect of the $\alpha5\beta1$ inhibitor on glioma invasion, suggesting that the $\alpha5\beta1$ integrin promotes tumor development through interactions with microglia [52].

As new novel strategies are being developed to target GAMs, recent reports suggest the existence of a therapeutic window when such approaches can be utilized. It seems that microglia/macrophage functional impairment may occur relatively late in the course of glioma growth, suggesting that early intervention to target these cells may provide the best therapeutic opportunity [53, 54].

6. Conclusion

GAMs appear to be a heterogeneous cell population with diverse roles in gliomatogenesis. These cells are actively recruited by gliomas and participate in tumor growth, invasion, angiogenesis, and local immunosuppression. A better understanding of their function will be helpful in developing novel therapies against malignant gliomas.

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Research Article

Microglia Play a Major Role in Direct Viral-Induced Demyelination

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Microglia are the resident macrophage-like populations in the central nervous system (CNS). Microglia remain quiescent, unable to perform effector and antigen presentation (APC) functions until activated by injury or infection, and have been suggested to represent the first line of defence for the CNS. Previous studies demonstrated that microglia can be persistently infected by neurotropic mouse hepatitis virus (MHV) which causes meningoencephalitis, myelitis with subsequent axonal loss, and demyelination and serve as a virus-induced model of human neurological disease multiple sclerosis (MS). Current studies revealed that MHV infection is associated with the pronounced activation of microglia during acute inflammation, as evidenced by characteristic changes in cellular morphology and increased expression of microglia-specific proteins, Iba1 (ionized calcium-binding adaptor molecule 1), which is a macrophage/microglia-specific novel calcium-binding protein and involved in membrane ruffling and phagocytosis. During chronic inflammation (day 30 postinfection), microglia were still present within areas of demyelination. Experiments performed in *ex vivo* spinal cord slice culture and *in vitro* neonatal microglial culture confirmed direct microglial infection. Our results suggest that MHV can directly infect and activate microglia during acute inflammation, which in turn during chronic inflammation stage causes phagocytosis of myelin sheath leading to chronic inflammatory demyelination.

1. Introduction

Microglia are specialized macrophages of the CNS that constitute 5–20% of total glial cells in rodents, depending on the specific neuroanatomical region of the CNS. Microglia are distinguished from neuron as well as glial cells, such as astrocytes and oligodendrocytes, by their origin, morphology, gene expression pattern, and function. While neuron and conventional glial cells are neuroectodermal in origin, microglia are of haematopoietic origin and act as primary responding cells for pathogen infection and injury like monocytes/macrophages in peripheral tissues. Microglia exhibit several features that distinguish them from other populations of macrophages, such as their "ramified" branches that emerge from the cell body and communicate with surrounding neurons and other glial cells. Microglia can rapidly respond to infectious and traumatic stimuli and adopt a "phagocytotic" nature. Activated microglia are known to produce many proinflammatory mediators including cytokines, chemokines, reactive oxygen species (ROS), and nitric oxide which mainly contribute to the clearance of pathogens or infections. However, prolonged or unwarranted microglial cell activation may result in pathological forms of inflammation which can lead to several neuroinflammatory conditions of the nervous system. Microglia-mediated innate immune response in the CNS is now considered to be potentially one of the major pathogenic factors in a number of CNS neuroinflammatory diseases that lack

the prominent leukocytic infiltrates of adaptive immune responses [1]. Neuroinflammation is associated with many neurodegenerative diseases, including Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), and multiple sclerosis (MS) [2]. While AD, PD, and ALS are commonly known to be neurodegenerative disease with underlying neuroinflammatory mechanisms, MS is one of the major chronic inflammatory CNS diseases in humans with heterogeneous (chronic/remitting) clinical presentations and course [3, 4].

MS is believed to be an autoimmune inflammatory demyelinating disease in which exposure of genetically predisposed people to environmental factors triggers a breakdown in T-cell tolerance to myelin antigens. Demyelination is a complex process, and while the precise mechanisms of this pathology are unclear, inflammatory demyelination is thought to be the result of adaptive immune-mediated responses to myelin antigens in the myelin sheaths of axons and/or in the myelin-forming oligodendrocytes. Most studies have focused on the pathogenic role of myelin-specific $CD4^+$ T cells because of the relatively strong association of susceptibility to MS with major histocompatibility complex (MHC) class II alleles [5, 6]. There is also increasing recognition of the potential importance of $CD8^+$ T cells in the pathogenesis of demyelination [7, 8]. However, the contribution of innate immune cells in mediating MS pathogenesis has been recently gained attention, as several studies demonstrated the role of various innate immune cells in mediating MS pathogenesis, in particular, the potential anti-inflammatory or proinflammatory function of microglial cells along with its physical interaction with myelin [9–11]. For long time, microglia were known to be present in the chronic inflammatory demyelinating plaque to remove myelin from the dead sick neuron in MS patients but the emerging recognition of microglia as CNS resident immune cells and their role in CNS health and diseases stimulated substantial efforts to redefine the role and function of microglia in the regulatory mechanisms of demyelination.

MS is best studied in some experimental models such as experimental autoimmune encephalitis (EAE), Theiler's murine encephalomyelitis (TMEV), and mouse hepatitis virus- (MHV-) induced neuroinflammation. Virtually, all types of adaptive immune response have been proposed to play important roles in the pathogenesis of EAE [4, 12], TMEV [13], and a neurotropic strain of mouse hepatitis virus (MHV); MHV-JHM [14, 15], mimicking the pathogenesis of the MS.

Upon intracranial (i.c.) infection of neurotropic MHVs, acute meningoencephalitis (with or without hepatitis) is the major pathologic process (see Supplementary Figure 1 available online at http://dx.doi.org/10.1155/2013/510396) [16]. Natural and genetically constructed recombinant MHV strains (generated by targeted RNA recombination) with differential pathological properties were used in several studies to understand the mechanisms of demyelination and concomitant axonal loss [17–20]. The outcome and degree of MHV-induced disease are dependent on several factors, including the age and strain of the mouse, the strain of MHV, and the route of virus inoculation. Even very closely related strains of MHV differ in pathogenic properties. Some strains of MHV are purely hepatotropic (e.g., MHV-2) [21]; some are primarily neurotropic (e.g., JHM, MHV-4, an isolate of JHM) [15, 22]; while others (e.g., MHV-A59 and MHV3) [16, 23] are both hepatotropic and neurotropic. Viral titer reaches its peak at days 3 and 5 postinfection (p.i.) [21]. Infectious virus is cleared within the first 10–14 days; however, at this time mice begin to develop demyelination, either clinical or accompanied by chronic hind limb paralysis [16, 24]. Both MHV-JHM and MHV-A59 cause inflammatory demyelination in the brain and spinal cord whereas MHV3 only causes vasculitis [23, 25]. It was formerly believed that in primary MHV-induced demyelination neuronal axons remain relatively preserved. Recently, it has been shown that axonal damage is, in large part, immune mediated in MHV-infected mice and occurs concomitantly with demyelination. Concurrent axonal loss and demyelination have recently also been observed with S protein recombinant demyelinating strain-infected mouse spinal cord [20].

Evidence from highly neurovirulent JHM strains of MHV suggests that MHV-induced demyelination is primarily immune mediated [26, 27]. Clearance of infectious virus is mediated by both cytolytic and cytokine-mediated mechanisms and microglia, and T cells modulate pathologic changes. Demyelination can be prevented in JHM-infected lymphocyte-deficient ($RAG^{-/-}$) mice [28]. However, demyelination will occur upon transfer of splenocytes from immunocompetent mice to $RAG^{-/-}$ mice [28]. It has also been shown by depletion and transfer studies in the JHM model that $CD8^+$ T cells can induce demyelination. These studies suggest that an intact adaptive immune system is required to promote demyelination in JHM-MHV infection. Contrary to these findings, demyelination, induced by MHV-A59, has been shown to develop in adult immunocompromised mice lacking B and T cells [29]. It has also been demonstrated that the depletion of $CD4^+$ or $CD8^+$ T cells after the acute stage of infection does not reduce demyelination [28, 30]. Indeed, MHV-A59 or its isogenic spike protein (host-attachment protein) recombinant strain, RSA59 [19, 20, 31], induces a MS-like disease in mice mediated by microglia, along with a small population of T cells. The mechanism of demyelination is at least, in part, due to macrophage-mediated myelin stripping, with some direct axonal injury as well as without involving the conventional $\alpha\beta$T cells. In our current studies, we have used RSA59 infection *in vivo*, *in vitro,* and *ex vivo* as a model to understand whether MHV can directly infect CNS resident microglia and the mechanism of microglial activation in the induction of chronic demyelination.

2. Material and Methods

2.1. Ethics Statement. Use of animals and all experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee at the Indian Institute of Science Education and Research Kolkata and Indian Institute of Science, Bangalore India. Animal protocols adhered to the guidelines of the CPCSEA, India.

2.2. Virus. RSA59 an isogenic recombinant demyelinating strain of MHV-A59, where the spike gene (encodes virus host-attachment protein), was exchanged by MHV-A59 spike gene only in the background of MHV-A59 gene by targeted RNA recombination as described in our previous studies [18, 19]. This recombinant strain also expresses enhanced green fluorescence protein (EGFP) [32] for easy detection of viral particle by EGFP fluorescence.

To engineer the targeted recombinant strains, molecularly cloned vector pMH54 [33, 34], which contains the entire 3′ end of the genome from MHV-A59, was used for construction of the recombinant viruses. RSA59 and RSMHV2 are isogenic except the spike protein. RSA59 strain is expressing the MHV-A59 spike in the MHV-A59 background, whereas RSMHV2 strain is expressing the MHV-2 spike in the MHV-A59 background [18]. Furthermore, EGFP gene was inserted into the MHV genome in place of the nonessential gene 4 in both RSA59 and RSMHV2 [19].

In order to replace gene 4 with the EGFP gene, pMH54 was modified by the introduction of a SalI site 42 nucleotides downstream of the intergenic sequence for gene 4a and a NotI site 102 bp upstream of the stop codon for gene 4b, using the Quick Change site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). (These are coding-silent nucleotide changes.) The coding sequence of EGFP was cleaved from the pEGFP-N1 vector (Clontech, Palo Alto, CA, USA) using SalI and NotI and inserted in the place of the SalI/NotI fragment of pMH54. The resulting plasmid contains 760 bp of non-MHV sequence, including the 722-bp EGFP open-reading frame, replacing the entire gene 4a and the rest 213 bp of gene 4b.

Previous studies reported with JHM strain revealed that the interruption of the ORF 4 did not alter the neurovirulence neither *in vivo* nor the replication *in vitro* [35]. Targeted recombination was used to select MHV isolates with stable and efficient expression of the gene encoding EGFP to facilitate the *in vivo* detection of virus in the mouse CNS as well as to trace the viral entry and spread in tissue culture. The viruses replicated with similar kinetics as wild-type virus both in tissue culture and in the mouse CNS. They caused similar encephalitis and demyelination in animals as the wild-type virus or their recombinant strains; however, they were somewhat attenuated in virulence [19].

2.3. Inoculation of Mice. Four-week-old, ten MHV-free, C57BL/6 (B6) mice (Jackson Laboratory, obtained from IISc, Bangalore, India) were inoculated intracranially with 50% LD_{50} dose of RSA59 strain (20,000 PFU) as described previously [19, 32]. Mice were monitored daily for signs of disease. Three mock-infected controls were inoculated similarly but with an uninfected cell lysate at a comparable dilution. Three mice were sacrificed in between days 5, 6, or 7 (period for peak of inflammation postinfection for routine paraffin-based histopathological analysis), and the other three were used for frozen sections. The rest of the infected mice were sacrificed at day 30 postinfection for routine paraffin-based histopathological analysis. Cervical, thoracic, and lumbar regions of spinal cord were successively processed, and 4 quadrants (dorsal/posterior column, anterior column, and two anterior horns) from two separate sections of each spinal cord level were examined.

2.4. Histopathology. At 5, 6, or 7 and 30 days postinfection, brain and spinal cord tissues were harvested from both mock infected and RSA59-infected mice. For routine paraffin sectioning, brain and spinal cord tissues were postfixed in 4% PFA for overnight. Fixed tissues were processed and 5 micron thin sections were prepared for routine CNS pathology, whereas frozen sections tissues were postfixed with 4% PFA for 4–6 hours and then transferred in 4% sucrose solution for 4–6 hours and in 20% sucrose solution for 16–24 hours and mounted in Cryomatrix (Thermo Shandon). Ten micron thin sections were prepared for frozen tissue immunofluorescence. The paraffin-embedded tissue sections were stained with hematoxylin and eosin (H&E) to determine the inflammation, whereas Luxol Fast Blue (LFB) staining was used to detect the loss of myelin sheath. All slides are coded and read in blind manner.

2.5. Immunohistochemical Analysis. Serial sections from brain and spinal cord were stained by the avidin-biotin-immunoperoxidase technique (Vector Laboratories) using 3,3-diaminobenzidine as substrate and a 1 : 100 dilution of anti-Iba1 (Wako, Richmond, VA, USA), 1 : 100 dilution of anti-CD45 (LCA; leukocyte common antigen, LY-5, BD Pharmingen), anti-Iba1 (Wako, Richmond, VA, USA), or CD3 (Dako; Carpinteria, CA, USA), and 1 : 20 dilution of monoclonal antibody directed against the nucleocapsid protein (N) of MHV-JHM (monoclonal antibody clone 1-16-1 (kindly provided by Julian Leibowitz)) as primary antibodies. Control slides from mock-infected mice were incubated in parallel.

2.6. Immunofluorescence and Fluorescence Microscopy. Cryosections from the spinal cord tissues were washed with PBS at room temperature in a humidified chamber, incubated for 10 min at room temperature with 1 mg/mL $NaBH_4$ in PBS to reduce autofluorescence, washed, incubated for 1 h at room temperature with 1 M glycine in PBS to reduce nonspecific cross-linking, and then washed subsequently with PBS, PBS with 0.5% Triton X-100 (TX), and PBS with TX and 2% goat serum (GS). The sections were incubated overnight at 4°C with a 1 : 100 dilution of a rabbit anti-Iba1 antibody diluted in PBS with TX and GS, washed, and then incubated with a secondary antiserum diluted into PBS with GS for 2 hrs at 37°C. All incubations were carried out in a humidified chamber. Viral antigen was detected by EGFP in a fluorescein isothiocyanate channel [19]. Control slides were incubated in parallel with preimmune rabbit sera, and sections from mock-infected mice were incubated with secondary antibodies only. Tissue sections were sequentially washed with PBS plus TX and with PBS and mounted and visualized by IX-81 fluorescence microscopy with a 40x UPlanApo objective, with the iris diaphragm partially closed to limit the contribution of out-of-plane fluorescence, and with filter packs suitable for green fluorescence and red fluorescence. Images were acquired with a Hamamatsu

Orca-1 charge-coupled device camera and Image-Pro image analysis software (Media Cybernetics, Silver Spring, MD, USA).

2.7. Transmission Electronic Microscopic Observation for the Accumulation of Microglia in the Demyelinating Plaque during Chronic Inflammatory Stage of RSA59 Infection. To further characterize the presence of microglia in the chronic demyelinating plaque at the ultrastructural level, mice were anesthetized, perfused with 4% PFA, and spinal cords from mock-infected and RSA59-infected were harvested and fixed overnight in 2% glutaraldehyde as described earlier [20]. Samples for transmission electron microscopy (TEM) were postfixed with 1% osmium tetroxide, dehydrated, and flat-embedded in Poly-Bed 812 epoxy resin (Polysciences). Half micrometer thick sections were cut from the lesional epicenter, stained with toluidine blue, and examined by light microscopy. Ultrathin TEM sections (600 Å) were cut from representative foci of demyelination from the toluidine blue-stained semithin sections and mounted on 200 mesh copper grids, stained with uranyl acetate and bismuth subnitrate, and viewed under a JEOL JEM 1010.

2.8. Ex Vivo Slice Culture. Four-week-old, MHV-free, C57 BL/6 were perfused transcardially with sterile PBS. Spinal cord was harvested and washed with PBS containing 1% penicillin/streptomycin (Pen/Strep). The spinal cord was then embedded in 2% agarose mould, and 200 micron thick cross-sections were prepared by Vibratome (Leica Vibrating Blade Microtome; VT1200S). The slices were washed twice with PBS containing 1% Pen/Strep. The slices were then transferred to a 24-well plate with one slice in each well. 500 μL of DMEM containing 10% FBS, 1% Pen/Strep, and 1% L-Glutamine were added in each well and incubated overnight with 5% CO_2.

2.9. Viral Infection and Staining of Spinal Cord Slices. After 24 hrs of explantation, slices were infected with RSA59 at 20,000 PFU (Half of the LD_{50} dose) in low serum (2%) containing medium for 1 hr and then washed with PBS to remove the unbound viruses, and 10% serum containing medium were added to the infected culture and the cultures were maintained for 72 hrs. At 24 hrs, 48 hrs, and 72 hrs of postinfection, slices were processed for immunostaining with anti-Iba1 antibody, and EGFP fluorescence was used to detect viral antigen. Briefly, at different times postinfection slices were washed gently with PBS and fixed with 4% PFA for 2 hours. Postfixed slices were washed with PBS, permeabilized with 0.5% Triton X-100 for 5 mins, and blocked with 1% goat serum for 1 hr at RT followed by overnight incubation with anti-Iba1 antibody. For better staining, next day antibody solution was replaced with fresh antibody and incubated at 4°C for additional 16–20 hrs. Slices were washed to remove any unbound antibody and then labelled with TRITC conjugated goat anti-rabbit IgG for 16 hrs. Labelled slices were then washed to remove any unbound fluorescent tagged antibody and then mounted in Vectashield (Vector Laboratories, CA, USA with DAPI and observed in Zeiss Confocal Microscope (LSM710). Images were acquired and processed by using Zen2010 software (Carl Zeiss).

2.10. Isolation of Mixed Glial Cells from Neonatal Mice Brain. Primary cultures of mixed glia from day 0 to day 3 newborn mice were prepared as described previously [36]. Briefly, following the removal of meninges, brain tissues were minced and incubated in a rocking water bath at 37°C for 30 min in Hanks Balanced Salt Solution (HBSS, GIBCO) in the presence of 300 μg/mL of DNaseI (Sigma) and 0.25% trypsin (Sigma). Enzyme-digested-dissociated cells were triturated with 0.25% of fetal calf serum (FCS), followed by a wash and centrifugation (300 ×g for 10 min). The pellet was resuspended in HBSS, passed through a 70 micron nylon mesh, followed by a second wash and centrifugation (300 ×g for 10 min). Following dilutions with astrocyte-specific medium (Dulbecco's essential medium containing 1% penicillin-streptomycin, 0.2 mM l-glutamine, and 10% FCS), cells were plated and allowed to adhere for 1 day in a humidified CO_2 incubator at 37°C. After 24 hrs, any nonadherent cells were removed and fresh astrocyte-specific medium was added. Adherent cells were maintained in astrocyte-specific medium for 10 days.

2.11. Isolation and Enrichment of Microglia from Mixed Glial Culture. After establishment of the mixed glia culture, feeding was stopped for 10 days to allow for significant microglial growth on top of the astrocyte monolayer. The microglia population peaked at 12–14 days in these cultures. To remove any cells adherent to the astrocyte monolayer, microglia-enriched cultures were thoroughly agitated in an orbital incubator shaker (200 rpm for 40 min at 37°C). Immediately following agitation, all cells suspended in the culture medium were collected and centrifuged at 300 ×g for 5 min at 4°C. The cell pellet was resuspended and diluted with fresh astrocyte-specific medium bringing the cells to a final concentration of 8×10^5 cells/mL; 1 mL was added to each well of a two-well CC2-treated chamber slide (specifically made for primary cell culture; NUNC) or 2 mL/well of a six-well plate. After 30 min, any non-adherent cells were discarded and adherent cells were maintained in fresh astrocyte-specific medium until infected with a medium change every 3-4 days.

2.12. Characterization of Microglia in a Mixed Glial Culture. To examine different cell types in a given culture, primary antibodies directed against cell-specific antigens were used to determine the presence and/or purity of each of the major glial cell types as described previously. Microglia were labelled with biotinylated anti-mouse CD11b (Chemicon, diluted 1 : 100 in F-12 nutrient medium) followed by Cy3-streptavidin (Jackson Immunoresearch, diluted 1 : 200 in F-12). Astrocytes were labelled with polyclonal rabbit anti-glial fibrillary acidic protein (anti-GFAP; DAKO) followed by either goat anti-rabbit Alexa488 (Molecular Probes), Cy2, or FITC (Jackson Immunoresearch) secondary antibodies. Before processing for double-label immunofluorescence microscopy, cells were washed in F-12 nutrient medium. Cells were incubated with primary antibodies to the surface

markers CD11b at room temperature followed by three 2 min washes with F-12. Cells were then incubated with fluorescently coupled secondary antibodies for 30 min followed by three washes with PBS containing Ca^{++}/Mg^{++}. Surface-labelled cells were fixed for 10 min in 4% paraformaldehyde followed by PBS washes, permeabilized with PBS/TX (PBS with Ca^{++}/Mg^{++}, 0.5% Triton-X) for 5 min, and successively washed with PBS/TX/GS (PBS with Ca^{++}/Mg^{++}, 0.5% Triton-X, 2.0% normal goat serum) three times for 5 min each. Cells were incubated for 30 min with the astrocytic marker GFAP, washed three times with PBS/TX, labelled with an appropriate secondary antibody, and stained with DAPI (1 : 500 diluted in PBS without Ca^{++}/Mg^{++} from 5 μg/mL stock solutions) for 5 min. Cells were then washed, mounted using Vectashield (Vector Laboratories), and visualized by fluorescence microscopy (Olympus I X-80) with a 20 PlanApo objective (1.0 numerical aperture). Images were acquired with a Hamamatsu ORCA CCD Camera and data were analyzed by using Image-Pro software.

2.13. Infection of Primary Microglial Cells with RSA59. On day 2 after seeding, neonatal microglial cultures were infected at a multiplicity of infection (MOI) of 2 : 1 with RSA59 or mock-infected with noninfected cell lysate. After allowing viral adsorption for 1 hr, cells were washed and placed in fresh media without virus. At 6, 12, and 24 hrs after infection, cultures were examined by microscopy for EGFP fluorescence.

2.14. RSA59 Growth Curve. Confluent monolayers of L2 cells were infected with undiluted and 1 : 2 diluted culture supernatant collected from the *in vitro* infected microglia and incubated for 1 hr at 37°C. Following adsorption, the cells were washed with Tris-Buffer saline 3 times and then fed with DMEM with 20% FBS mixed with 0.4% agarose for overlaying. 48 hours postinfection, culture was subjected for plaque count [32].

3. Results

3.1. CNS Pathology of RSA59. To confirm the RSA59-induced CNS inflammation, brain and spinal cord sections from day 7 (peak of inflammation) and day 30 (peak of demyelination) postinfected mice were stained with H&E or LFB and examined. RSA59-induced meningitis (Supplementary Figure 1(a)), and encephalomyelitis (accumulation of inflammatory cell and perivascular cuffing) (Supplementary Figures 1(b) and 1(c)) were observed as shown previously [18, 19] (Supplementary Figure 1; these data are partly published but for the ready information compiled in one figure.). To characterize inflammatory cell types, brain sections from day 7 postinfection were stained immunohistochemically with anti-CD45 (leukocyte common antigen (LCA)), anti-CD11b and/or anti-Iba1 (macrophage/microglial marker), or anti-CD3 (pan T-cell marker) (data not shown). The majority of inflammatory cells in RSA59-infected brains were immunoreactive for both LCA (Supplementary Figure 1(d)) and CD11b (Supplementary Figure 1(e)) and Iba1 (Supplementary Figure 1(f)). Some CD3-stained infiltrating T cells were also found (data not shown), although nonspecific background staining of neurons with available anti-CD3 antibodies made quantification difficult. No CD4- and CD19-positive cells but few CD8-positive cells were observed in the inflamed brain and spinal cord sections in RSA59-infected mice (data not shown). Demyelination was observed by LFB staining as early as day 7 as examined (Supplementary Figure 1(h)) and it reaches its peak at day 30 postinfection (Supplementary Figure 1(i)) as observed earlier [20, 31]. LFB-stained spinal cord section showed no myelin loss (Supplementary Figure 1(g)). Together, the data indicate that RSA59 causes meningoencephalitis and demyelination. CNS inflammation consists of a mixed population of inflammatory cells, predominantly macrophages/microglia as well as a smaller population of T lymphocytes as shown previously [17, 20, 37].

3.2. Direct Infection of CNS Resident Microglia in RSA59 Infection during Acute Inflammation. Previously, it has been demonstrated that neurotropic strains of MHV can directly infect different neural cell types [16, 19, 38, 39] but there is no evidence whether neurotropic strain can directly infect microglia or only acquire activity indirectly due to the infection of other neural cell types. In order to determine the tropism of RSA59 in CNS resident microglia, four-week-old, MHV-free, C57BL/6 (B6) mice (Jackson Laboratory) were inoculated intracranially with RSA59. Mice were sacrificed at the peak of inflammation (day 6), and the spinal cord sections were prepared for cryostat sectioning. Since RSA59 expresses EGFP, viral antigen was viewed directly by fluorescence microscopy. Identification of CNS resident microglia was performed by using Iba1 as a specific marker for microglia/macrophages [40]. While Iba1 immunofluorescence was observed in both gray and white matter, double fluorescence/immunofluorescence demonstrated dual labelling of EGFP (viral antigen) positive Iba1 positive microglia/macrophages were present only in the white matter of RSA59 infected mice (Figure 1). In the white matter, all the microglia (Iba1-positive) were not infected as shown by arrowheads (Figures 1(e) and 1(f)). In the control mock infected spinal cord section, no double fluorescent labelled cells were observed as expected (data not shown).

3.3. Trafficking of Microglia from Gray Matter to the White Matter with Different Days of Postinfection of RSA59. Previous studies demonstrated that with time of postinfection viral antigen spread from gray matter to white matter [19] in the infected mice. This phenomenon is more prevalent in the spinal cord of infected mice as gray and white matter is clearly separated from white matter. Immunostained section demonstrated that the viral antigen is localized both in gray and white matter at day 7 postinfection (Figure 2(a)). At day 30 viral antigen is below the detection level, more specifically after day 10 postinfection (as observed) viral antigen is below the detection limit (data not shown) as discussed previously [18, 19]. To determine whether microglia also follow the trajectory of the viral spread at days 7 and 30 postinfection,

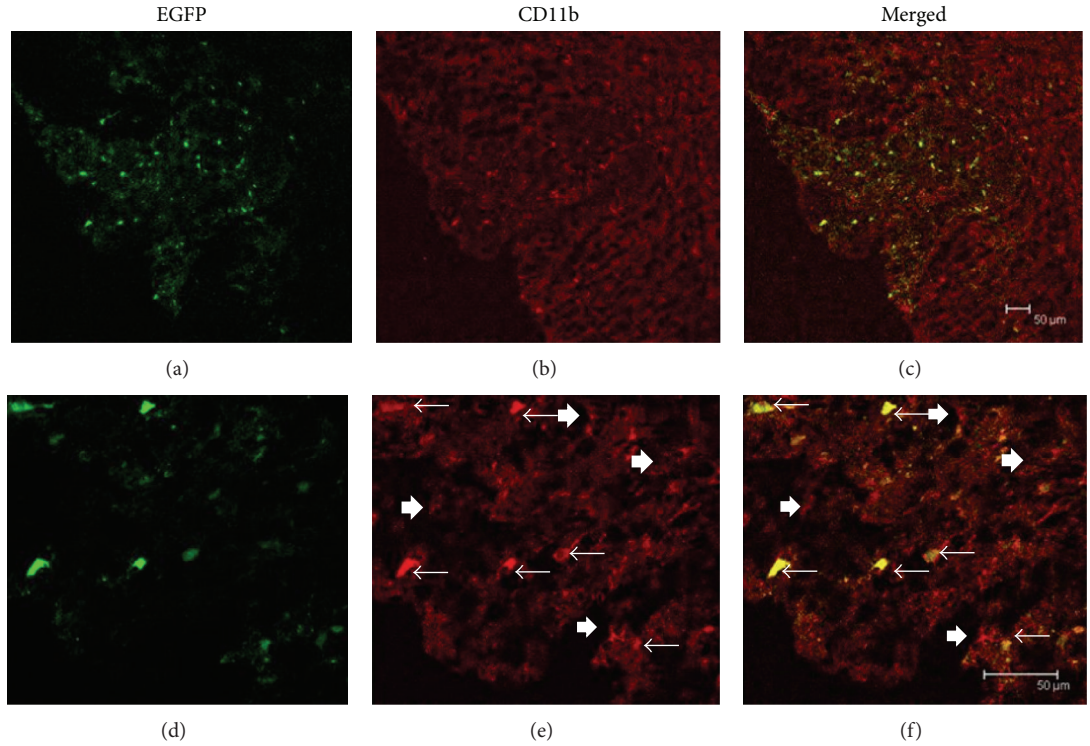


Figure 1: Colocalization of EGFP-expressing cells (positive for viral antigen) and microglia/macrophages (CD11b) in spinal cord white matter (dorsal columns) of RSA59-infected mice. RSA59-infected mice (low magnification (a–c); high magnification (d–f)). EGFP fluorescence (green) identifies virus-infected cells (a, d) and anti-CD11b (red fluorescence) detects microglia/macrophages (b, e). Merged images (c, f) in RSA59-infected spinal cord show colocalization of EGFP in CD11b-positive cells in spinal cord white matter. Arrows mark CD11b-positive cells that are infected (EGFP-positive), whereas arrowheads mark CD11b-positive cells that are not infected (EGFP-negative).

spinal cord tissues sections were immunostained with anti-Iba1 antibody. While Iba1-positive cells were scattered in both gray and white matter at day 7 (Figure 2(b)), at day 30 postinfection Iba1-positive cells are mainly localized to the white matter (Figures 2(c) and 2(e)). At day 30, most of the Iba1 positive cells are present in the inflammatory demyelinating plaques as observed in the LFB-stained serial sections (Figures 2(d) and 2(f)). If any Iba1-positive cells are present in the gray matter, they are mainly reverted to their quiescent stage (as observed by morphology). Time-dependent trafficking of Iba1-positive cells demonstrate that microglia follow the track of viral antigen spread.

3.4. High-Resolution Electron Micrographic Analysis Confirms the Presence of Vacuolated Microglia with Engulfed Myelin Debris in Demyelinating Plaque. Previously microglial accumulation was observed in the demyelinating plaque of RSA59 with an emphasis on the stripping of the myelin sheath [20]. To reemphasize on the accumulation of microglia in the demyelination plaque during chronic phase of the inflammation at ultrastructural level, semithin sections were cut at 1 micron intervals from five infected mice at day 30 post infection. Semithin sections were stained with toluidine blue. Control mock-infected mouse spinal cord was used to evaluate for background fixation and/or postfixation artefacts (Supplementary Figure 2(a)). RSA59-infected spinal cords showed significant myelin loss and accumulation of phagocytotic microglia within plaques as observed earlier (Supplementary Figures 2(b) and 2(c)). Representative foci of demyelination were selected from semithin sections, and 600 Å ultrathin sections from Poly-Bed embedded blocks were processed for TEM. High-resolution TEM images show accumulation of large number of microglia with no basement membrane which is the characteristic features of microglia/macrophages (Supplementary Figure 2(e)). Multiple vacuoles with myelin fragments were seen within the cytoplasm of the microglia in the plaque (Supplementary Figure 2(f)). No such microglial accumulation was observed in the control mock infected mice at high-resolution TEM images (Supple Figure 2(d)).

3.5. RSA59 Can Infect Microglia in Ex Vivo Spinal Cord Explants Culture in Absence of Any Peripheral Inflammatory Cells. In vivo colocalization of Iba1 with EGFP-(viral antigen) positive cells demonstrated that RSA59 can directly infect microglia but that does not confirm that infected microglia were resident microglia because in intracranial (IC) inoculation blood brain barrier can be disrupted and blood monocytes/macrophages can migrate and acquire infection. To exclude this possibility, 200 micron thick explants cultures were prepared from the four-week-old, C57BL/6 mice spinal cord and infected with RSA59. At 24, 48 and 72 hrs of

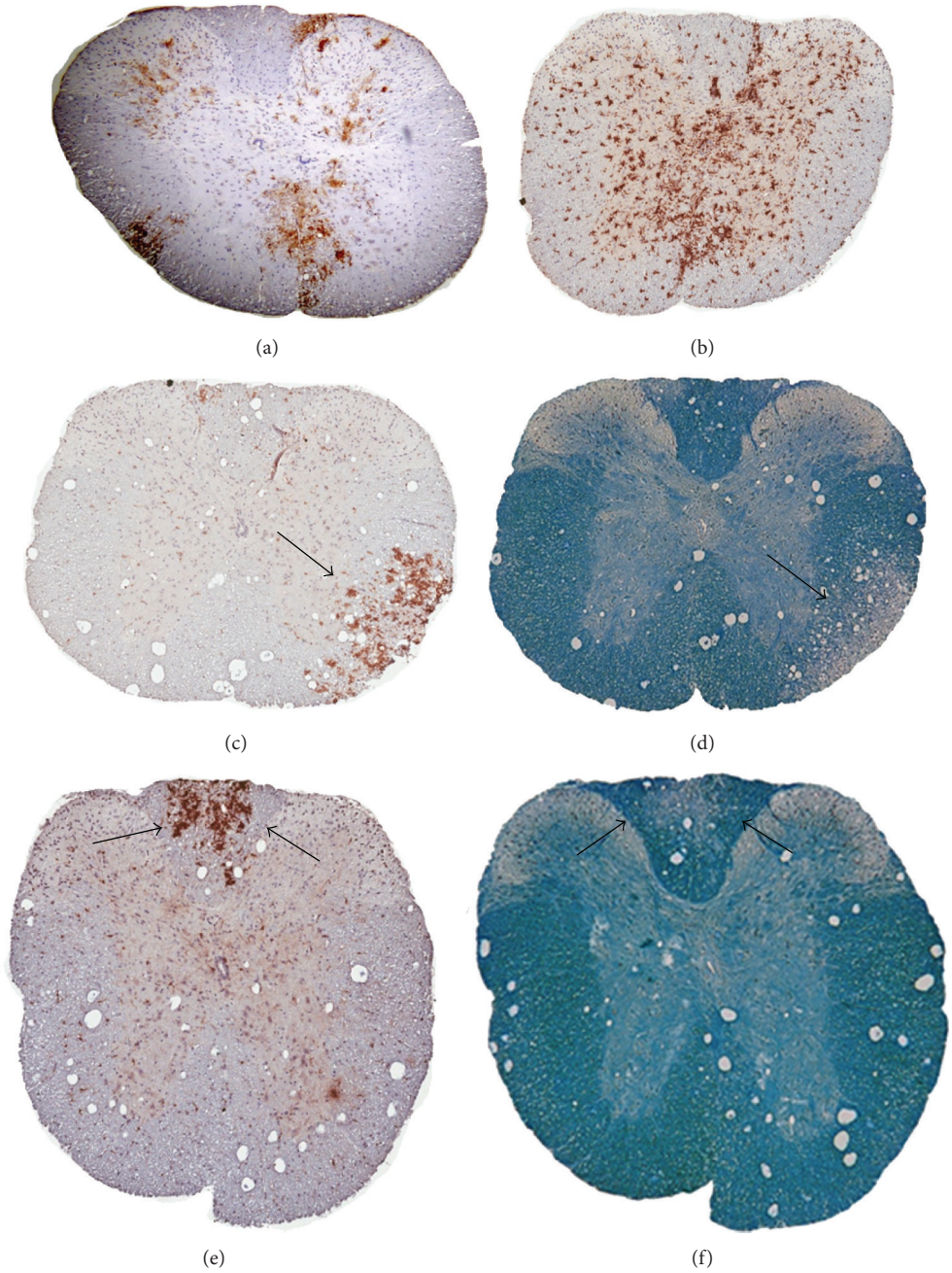


Figure 2: Distribution of viral antigen and microglia in the spinal cord section of RSA59-infected mice at different days post infection. Mice were inoculated intracranially with RSA59 or mock-infected and sacrificed at day 7 and day 30 postinfection. Spinal cord sections were stained with antinucleocapsid antiserum and/or anti-Iba1 antisera. At day 7 postinfection, viral antigen is present both in gray and white matter but predominantly in the white matter (a). Similarly, at day 7 postinfection, Iba1-positive cells are scattered throughout the gray and white matter with a tendency of white matter accumulation similar to viral antigen (b). At day 30 post infection majority of the Iba1 positive cell-(c, e) are present in the demyelination plaque (LFB-stained section (d, f)). Day 30 spinal cord sections are shown from two different RSA59-infected mice. Arrows show accumulation of Iba1 in (c, e) and demyelinating plaque in (d, f). Arrow, indicate Iba1-positive cells in the demyelination plaque.

postinfection explants, slices were processed for fluorescent-immunostaining with anti Iba1-antibody and EGFP fluorescence was used to detect viral antigen. Confocal microscopic observation of double label immunofluorescence slices demonstrated that in absence of any peripheral immune cell, RSA59 can directly infect resident microglia. At 24 hrs post infection very few microglia were positive for viral antigen (EGFP-positive) (Figure 3(c)) but the number of double positive cells was increased with time as examined in 48 and 72 hrs (Figures 3(g) and 3(k)) which demonstrated that

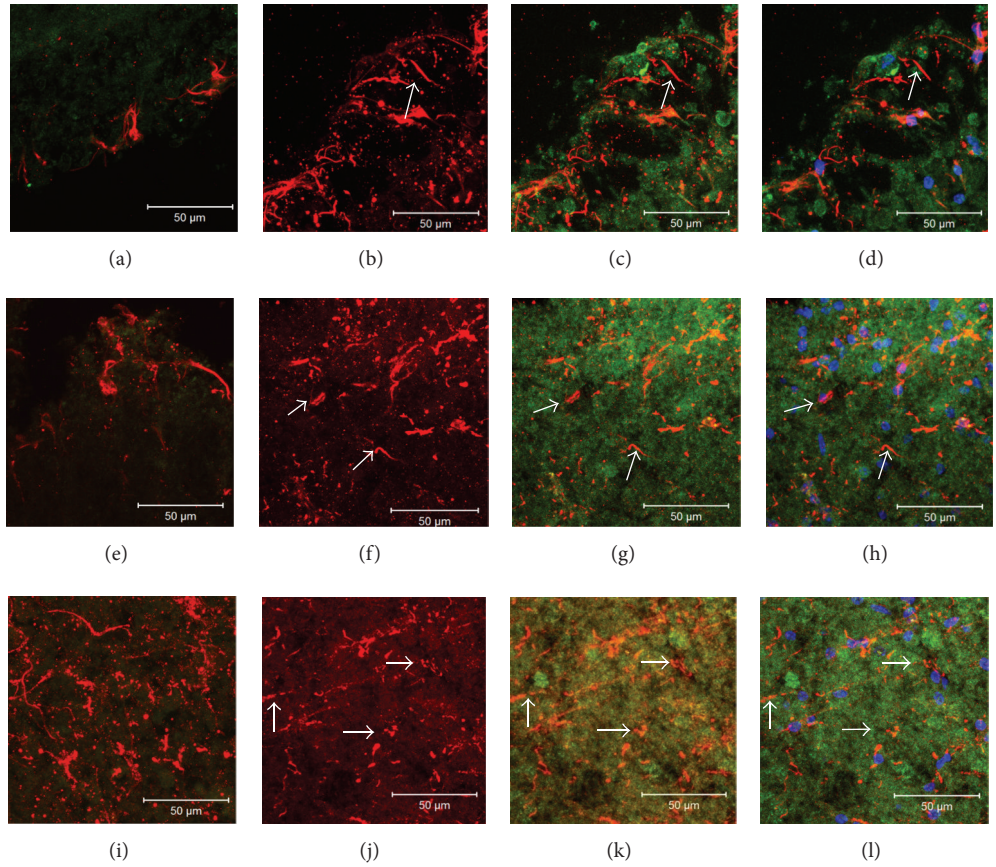
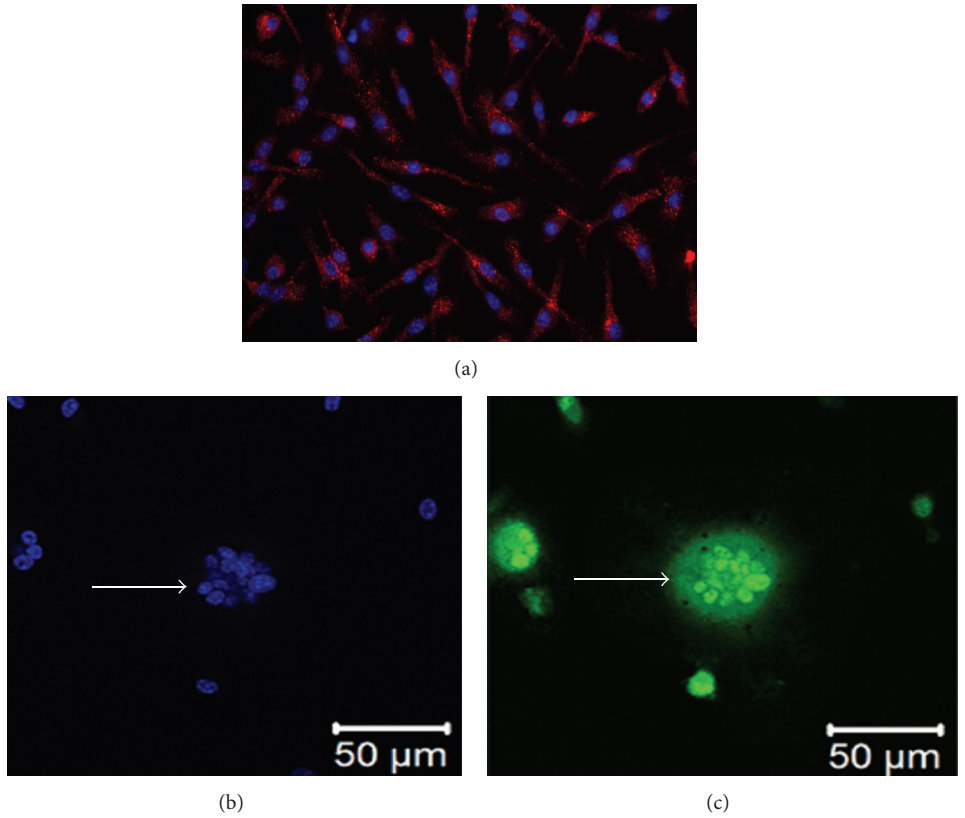


Figure 3: RSA59 can directly infect CNS resident microglia in the spinal cord explants culture in absence of any peripheral immune cells. Two hundred micron thick slice cultures were prepared from 4-week old C57BL/6 mice. 24 hrs after postexplantation, cultures were infected with 20,000 PFU/mL of RSA59 and incubated for 24 hrs, 48 hrs, and 72 hrs. Mock-infected cultures were also maintained in the same culture condition for the same period post explantation. 24, 48, and 72 hrs postinfection mock and RSA59-infected slices were stained with Iba1. Iba1 staining (red fluorescence) shows CNS resident microglia in mock-infected (a, e, i) and RSA59-infected slice culture (b, f, j). Merged images (c, d, g, h, k, l) show colocalization of EGFP in Iba1-positive cells of RSA59 infected spinal cord white matter. EGFP fluorescence (green) identifies virus-infected cells, anti-Iba1 (red fluorescence) detects microglia and DAPI (blue fluorescence) stains nucleus in the merged images.

CNS resident microglia can directly acquire infection and become activated (by morphological analysis as number of processes increased and enlarged). Arrowheads in Figures 3(b), 3(c), 3(d), 3(f), 3(g), 3(h), 3(j), 3(k), and 3(l) showed that some of the resident microglia did not get infection. Control noninfected explant cultures were also immunolabeled with anti-Iba1 antibody (Figures 3(a), 3(e), and 3(i)) as microglia *in vitro* in culture system behave like activated macrophages due to perturbation of the culture system. Figures 3(d), 3(h), and 3(l) show a merged image of EGFP (viral antigen; green), Iba1 (microglia; red), and DAPI (nucleus; blue) and demonstrate the presence of viral antigen in the cell cytoplasm of microglia.

Due to the thickness of the slices, clarity of the images was slightly compromised. RSA59 infection in *ex vivo* explant cultures demonstrated that in the absence of peripheral immune cells CNS resident microglia can directly be infected.

3.6. RSA59 Can Infect Neonatal Microglia Cells and Forms Syncytia. In order to determine whether RSA59 can infect microglial cells *in vitro* in absence of any neural cells, 2-day-old neonatal microglial cultures were infected at a multiplicity of infection (MOI) of 2 : 1 with RSA59 or mock infected with noninfected cell lysate. Microglia harvested in the cell suspension by the conventional shake-off method as described earlier [36] were 99 ± 0.5% positive for CD11b staining (Figure 4(a)). Very few GFAP (astrocyte marker) positive cells were observed in the isolated microglia culture (data not shown). At 0, 6, 12, and 24 hrs after infection, cultures were examined by microscopy for EGFP fluorescence. At 0 and 6 hrs, no fluorescence was observed in the infected culture but at 12 hrs bright fluorescence started to appear denoting the presence of viral antigen in the microglia. At 12 hrs postinfection, infected microglia demonstrated stressed morphology and started to fuse with the neighbouring cells, and at 24 hrs postinfection, most of the infected cells were involved into large syncytia formation (Figure 4(c)) which is a characteristic of some enveloped RNA viruses and more specifically characteristic of MHV-A59 (parental strain of RSA59), an enveloped demyelinating strain of MHV [41]. Nucleus of the syncytia was very obvious as shown in Figure 4(b) by DAPI



(a)

(b) (c)

Figure 4: Infection of microglia culture (free from astrocytes) with RSA59 demonstrates syncytia formation. Purified microglial cultures from the neonatal mixed glia were stained with anti-CD11b conjugated to Cy3 and anti-GFAP (astrocyte marker). Three different cultures were immunostained but the best representative culture is shown in (a). Immunostained culture demonstrates that majority of the isolated cells are microglia (positive for CD11b-Cy3) and negative for astrocytes (GFAP-negative). Purified cultures were infected with RSA59 at 2 : 1 M.O.I, and after 24 hrs postinfected cultures were observed under epifluorescence microscope. At 24 hrs postinfection, the majority of the cells were infected and most of the infected cells form syncytia (b, c). DAPI staining of infected cells shows the nucleus in the syncytia (b) and EGFP fluorescence demonstrates the RSA59 infection (c). Five culture plates were infected but the representative infected culture is shown here.

staining. *In vitro* experiment demonstrated that RSA59 can infect primary microglia in isolated culture and can also induce syncytia in primary microglia.

3.7. Viral Growth Curve. To demonstrate that the virus is replicating in the microglia, culture supernatant of infected microglia was assessed by routine plaque assay [32]. Routine plaque assay found very few plaques which were below the detection limit. But there, discrete syncytia was observed in the infected plates which denoted that the titer could be 30–50 PFU/mL.

4. Discussion

To understand the cellular mechanism of demyelination of neurotropic strain of MHV, prior studies in our laboratory have analyzed the detailed pathogenesis of recombinant MHV strain, RSA59 (demyelinating strain (DM)) and compared it with RSMHV2 (nondemyelinating strain (NDM)) that is isogenic except for the spike gene that encodes the virus-host-attachment spike glycoprotein [18, 20]. Both strains are capable of causing hepatitis, encephalitis, and meningitis. However, the two strains differ in their ability to induce subsequent demyelination and axonal loss [20]. Seven days post infection, RSA59 produces demyelination that is best observed in the spinal cord at day 30 postinfection (peak of inflammation). In contrast, RSMHV2 does not produce demyelination and only rarely demonstrates axonopathic changes in spinal cord white matter [20]. The inability of RSMHV2 to induce demyelination is due in part to a lack of transport of viral antigen (and the subsequent inflammatory reaction) to the white matter. Furthermore, *in vivo* and *in vitro* experiments demonstrate deficits in the ability of RSMHV2 to spread between neurons when compared to interneuronal spread by RSA59 [20]. RSA59-induced demyelination occurs in the setting of both axonal degeneration and macrophage mediated myelin stripping along intact axons [20]. While spike glycoprotein mediates spread of viral antigen to white matter through axonal transport, specific mechanisms leading to subsequent demyelination are not known. One plausible explanation is that MHV spreads

intra-axonally within gray matter and when it reaches the white matter, viral particles may need to spread directly into oligodendrocytes, astrocyte, and microglia, using the spike protein, and can induce local oligodendroglial dystrophy and inflammation. Viral antigen in white matter axons may be sufficient to trigger an inflammatory response via microglial activation. Infected and activated microglia due to its intrinsic nature of chemotaxis can recruit more microglia to the site of inflammation and strip myelin from the damaged axon and successively cause demyelination.

Our current *in vivo* studies support this hypothesis that RSA59 can infect CNS resident microglia. The migration and activation of numerous microglia to the white matter during acute inflammation and the retention of microglia in the chronic inflammatory plaque reinforce the hypothesis that CNS resident microglia can be recruited to the region of local CNS injury. Ultrastructural morphology of microglia containing multiple vacuoles with myelin fragments in the cytoplasm in the demyelinating plaque further substantiate that CNS resident activated microglia can mediate myelin stripping and can successively mediate demyelination.

As RSA59 spread intra-axonally, no colocalization was observed within the gray matter CNS resident microglia. If haematogenous propagation of peripheral monocytes/macrophages occurred to the CNS, one would expect more widespread distribution of activated microglia throughout the spinal cord which may not discriminate gray/white matter track. Furthermore, the delay in complete development of demyelination following partial resolution of encephalitis (up to 30 days after peak inflammation) documented in previous studies would not be expected [16, 18, 31]. Moreover, *ex vivo* colocalization of EGFP-positive cells with microglia confirmed that RSA59 can directly infect CNS resident microglia in absence of peripheral immune cells. *In vitro* infections of neonatal microglia demonstrate that RSA59 not only infects, but microglia can also forms syncytia which suggests that microglia supports RSA59 infection via cell-to-cell contact. Current combined *in vivo*, *in vitro,* and *ex vivo* explants culture studies established that the recruitment of microglia occurred from the CNS resident microglial pool rather than peripheral monocyte/macrophages.

Our current studies are focused on the understanding of the innate immune mechanism of CNS resident microglia activation and maturation to perform phagocytotic activity. Affymetrix microarray analyses for mRNA expression have revealed that expression of inflammatory mediators by MHV infected microglia, including chemokine and inflammatory cytokines. MHV infection of the mouse spinal cord was also associated with increased expression of genes involved in IFN signalling compared to mock-infected controls in the CNS. During chronic infection (day 30 postinfection), microglia are still present within areas of demyelination and microglia-associated inflammatory mediators are still produced which indicates that microglia are still active. Our results suggest that putative activated microglia and inflammatory mediators contribute to a local CNS microenvironment that eventually regulates viral replication and IFN-gamma production during acute phase of infection. Sequentially, IFN-γ can activate microglia by promoting phagolysosomes maturation and activation (engulfment of the myelin sheath) leading to demyelination. Affymetrix microarray data warrants further confirmation.

Viral infection in the CNS is classically recognized as inflammatory in nature, with meningeal perivascular and parenchymal infiltrates of peripheral leukocytes but RSA59 infection could be an exception where inflammation can proceed with CNS resident glial activation without involving the peripheral immune responses like Rabies virus infection [42], HIV infection [43], and prion diseases [44, 45]. In this perspective, it is tempting to speculate that the underlying mechanism of chronic myelin loss in MS could be a combination of persistence of myelin-related autoimmunogens that has escaped self-tolerance with persistence of activated CNS resident microglia which can mediate demyelination by phagocytised myelin.

Microglia are known for their innate immune function for long time but the role of microglia in chronic inflammation opens a new episode in the field of glial biology of neuroinflammatory diseases. The concept of chronic inflammation as opposed to acute inflammation is more relevant in the context of understanding other CNS diseases, more specifically neurodegenerative diseases like Alzheimer's disease, amyotrophic lateral sclerosis, Parkinson's disease, and Huntington's disease. These neurodegenerative diseases lack the prominent infiltrates of mononuclear cells but the underlying mechanism of inflammation could be through activation of CNS resident microglia. Activation of CNS resident microglia in the context of chronic neuroinflammation as one of the underlying mechanism of neurodegeneration warrants further study. Microglia as the prime components of an intrinsic CNS resident immune system become a major focus in cellular neuroimmunology and, therefore, in neuroinflammation.

5. Conclusions

It has been known for long time that in absence of conventional $\alpha\beta$T cells microglia play a major role in neurotropic MHV-induced demyelination but the mechanism of infection and route of infection were not very clearly known for long time. Our current microglial tropism studies revealed that RSA59, an isogenic demyelinating strain of MHV, can infect and activate CNS resident microglia, and microglia can help to mediate demyelination by engulfing myelin debris. RSA59-induced neuroinflammatory models are helpful in understanding direct CNS cellular injury and demyelination that does not require an intact adaptive immune system. Understanding the role of direct CNS resident microglial infection and activation will shed some light on the pathogenesis of CNS inflammatory disease, not only infectious diseases but also chronic CNS disorders. The vision of CNS-resident-microglia-driven neuroinflammatory responses in RSA59 with neuropathological consequences has extended the avenue to explore the contribution of microglia in chronic neuroinflammatory CNS diseases.

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Review Article

The Benefits and Detriments of Macrophages/Microglia in Models of Multiple Sclerosis

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The central nervous system (CNS) is immune privileged with access to leukocytes being limited. In several neurological diseases, however, infiltration of immune cells from the periphery into the CNS is largely observed and accounts for the increased representation of macrophages within the CNS. In addition to extensive leukocyte infiltration, the activation of microglia is frequently observed. The functions of activated macrophages/microglia within the CNS are complex. In three animal models of multiple sclerosis (MS), namely, experimental autoimmune encephalomyelitis (EAE) and cuprizone- and lysolecithin-induced demyelination, there have been many reported detrimental roles associated with the involvement of macrophages and microglia. Such detriments include toxicity to neurons and oligodendrocyte precursor cells, release of proteases, release of inflammatory cytokines and free radicals, and recruitment and reactivation of T lymphocytes in the CNS. Many studies, however, have also reported beneficial roles of macrophages/microglia, including axon regenerative roles, assistance in promoting remyelination, clearance of inhibitory myelin debris, and the release of neurotrophic factors. This review will discuss the evidence supporting the detrimental and beneficial aspects of macrophages/microglia in models of MS, provide a discussion of the mechanisms underlying the dichotomous roles, and describe a few therapies in clinical use in MS that impinge on the activity of macrophages/microglia.

1. Introduction

The central nervous system (CNS), consisting of the brain and spinal cord, is immune-privileged with access to leukocytes being limited. In several neurological diseases including multiple sclerosis (MS), however, significant infiltration of immune cells from the periphery into the CNS is observed. Demyelination and axonal degeneration are common consequences of CNS inflammation [1]. In addition to extensive accumulation of macrophages, the activation of microglia, the phagocytic cells of the CNS, is a common occurrence following neurological injury [2–6]. This review will discuss the roles of macrophages and microglia as evidenced in the common immune-mediated animal model of MS, experimental autoimmune encephalomyelitis (EAE), as well as in the two prominent demyelinating models of MS, cuprizone and lysolecithin injury.

2. Microglia and Macrophages

Microglia and bone marrow-derived macrophages are two genetically distinct myeloid populations [7, 8]. Microglia are the resident immune cells of the CNS and originate from erythromyeloid precursors in the embryonic yolk sac. In early gestation, these precursor cells differentiate into microglia and invade the developing neural tube [7, 9, 10]. In contrast, macrophages are derived from hematopoietic stem cells in the bone marrow. These cells differentiate into blood monocytes which circulate the peripheral vasculature and populate tissues such as the liver, lungs, and nonparenchymal areas of the CNS, including the meninges, choroid plexus, and perivascular space [11, 12]. In the healthy CNS, resting microglia are characterized by many ramified processes, surveying the parenchyma for any possible threats to neurons and macroglia. Under physiological conditions, bone marrow-derived monocytes do not contribute to the local microglia

pool [13, 14]. These observations suggest that microglia are sustained by local progenitors. Upon CNS injury, these cells become activated and take on an amoeboid shape, characterized by retracted processes. It is during this state of the CNS when bone marrow-derived macrophages also infiltrate the CNS and accumulate at the injury site, contributing to both further damage and tissue repair [11]. Macrophages within CNS lesion sites are difficult to distinguish from activated microglia, as both are amoeboid-shaped and express many of the same antigenic markers [15]. Due to difficulty in distinguishing these phagocytic cells, many authors refer to these cells collectively as macrophages/microglia.

Although there seems to be a spectrum of different types of macrophages/microglia, there are two main phenotypes that occur prominently in inflammatory lesions. These phenotypes are the classically activated "M1" cells and the alternatively activated "M2" cells [16, 17]. The following discussion is a simplified description as a more sophisticated discussion of these different subsets is beyond the scope of this review (refer to [2, 18]). The M1 macrophages/microglia are generally considered proinflammatory, as they are associated with the secretion of many proinflammatory cytokines including interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α). M1 cells also express the cell surface markers CD86 and CD16/32 and have inducible nitric oxide synthase (iNOS) activity [11]. The M2 macrophages/microglia on the other hand are anti-inflammatory in nature, as they are associated with the secretion of anti-inflammatory cytokines, such as IL-10. This subclass of macrophages/microglia can be identified by the expression of the mannose receptor (CD206) as well as the enzyme arginase 1 (Arg1). Most cells in the injured spinal cord were found to be of the M1 subset particularly at early stages of injury [19].

3. Roles of Macrophages/Microglia in CNS Injury

In the CNS of patients with Alzheimer's disease and MS patients, as well as in their models, a substantial infiltration of macrophages as well as an activation of resident microglia is prominent. This infiltration has been associated with disease severity and represents the predominant immune cell type present in MS autopsy studies [20, 21]. In addition, targeted depletion of CD95 ligand (CD95L), a protein thought to be important in the survival and migration of these cells into the injured CNS, reduced the representation of macrophages in an experimental rodent spinal cord injury model, correlating with enhanced locomotor recovery [22]. Furthermore, treatment with minocycline, an antibiotic that has been thought to reduce the activation of macrophages/microglia and the expression of inflammatory cytokines and matrix metalloproteinases (MMPs), reduced the amount of phagocytic cells in the lesion site, leading to a greater neurological recovery [23–25]. Popovich et al. [26] found that the depletion of macrophages using clodronate liposomes improved neural and motor recovery after experimental spinal cord injury. In addition to these detrimental effects observed *in vivo*, Kigerl et al. [19] showed the ability of M1-polarized macrophages to induce neuronal death. The mechanisms by which activated microglia kill neurons have been amply summarized [27] and include the elaboration of free radicals, proteases, and glutamate.

Despite these observations of detrimental roles of macrophages/microglia within the injured CNS, many studies have also reported beneficial roles of these innate immune cells. For instance, transplantation of macrophages into the injured CNS promoted the survival of neurons, functional recovery, and nerve regeneration [28]. In the study reporting enhanced nerve regeneration, activated macrophages in the crushed optic nerve were found to secrete a calcium-binding protein, called oncomodulin, which promoted neuron survival and axonal regeneration [29]. Macrophages/microglia have been demonstrated to produce other prorepair molecules, including brain-derived neurotropic factor (BDNF), IL10, and ferritin [30–33]. The latter molecule has been demonstrated to increase the proliferation and differentiation of oligodendrocyte precursor cells (OPCs). In mice expressing a mutated HSV-1 thymidine kinase (TK) gene controlled by the myeloid-specific CD11b promoter, administration of ganciclovir depletes cells of myeloid origin. In a study employing this technique, Barrette et al. [34] was able to show decreased axonal regeneration, increased myelin debris, and severely compromised locomotor recovery in CD11b-TK mice administered ganciclovir. This study demonstrates the reparative role that macrophages play in nerve injury. In mice deficient of CCR2, a receptor involved in recruiting macrophages to the lesion site, accelerated progression of Alzheimer's-like disease was demonstrated, with increasing cognitive impairments and amyloid deposition becoming evident [35, 36]. Another study depleted microglia and subsequently observed the formation and maintenance of amyloid plaques, suggesting that plaques can form independent of microglia [37]. In another study of Alzheimer's-like disease, CD11b-TK mice were again used to show that macrophages play an important role in clearing amyloid deposits [38].

From these studies, it is clear that macrophages/microglia can be both detrimental and beneficial in many different diseases affecting the CNS (Figure 1). To further highlight the dichotomy present in the roles of macrophages/microglia, the remainder of this review will discuss the functions of these cells in three animal models of MS. The reader should bear in mind that although these animal models have yielded a wealth of information concerning the biology of multiple sclerosis, such models represent an artificial mechanism of disease induction.

4. MS and Its Inflammatory Model, EAE

MS is a chronic, inflammatory neurodegenerative disease characterized by demyelination and remyelination in the majority of patients in the early phase of the disease. Clinical relapses are thought to be due to multifocal infiltration of immune cells, leading to loss of oligodendrocytes, demyelination, and axonal injury/loss. After a course of the relapsing-remitting part of the disease, most patients enter a phase of the disease characterized by progressive neurodegeneration, manifesting in irreversible disability in MS patients [39]. Demyelinating lesions are often found in the white matter

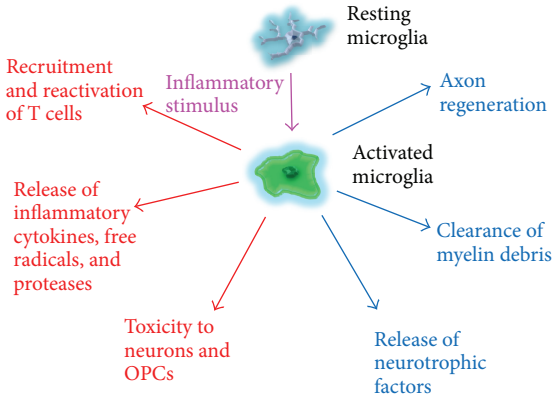

Figure 1: The dichotomy of macrophages/microglia is displayed. Macrophages/microglia have been shown to recruit and reactivate T cells in the CNS and release many detrimental molecules such as proteases, inflammatory cytokines, and free radicals. Through the latter molecules and other mechanisms, macrophages/microglia have been reported to contribute to toxicity to neurons as well as oligodendrocyte precursor cells. Conversely, they have also been observed to aid in axonal regeneration and remyelination as well as assist in the clearance of inhibitory myelin debris. In addition, macrophages/microglia have been shown to release a variety of neurotrophic factors. It can therefore be seen that macrophages/microglia possess an array of detrimental and beneficial functions, with the balance being dictated by the temporal and spatial specifications following CNS injury.

of the brain stem, spinal cord, optic nerve, and cerebellum [39, 40]. Activated microglia and macrophages are frequently observed in active MS plaques [1, 20, 21, 41].

A common animal model of MS is EAE. The disease is induced by immunization with myelin components, including myelin oligodendrocyte glycoprotein (MOG), myelin basic protein (MBP), and proteolipid protein (PLP) [42]. Most studies are carried out with mice immunized with a 35 to 55 residue MOG peptide emulsified in Freund's adjuvant supplemented with *Mycobacterium tuberculosis* extract. Mice may then be injected with pertussis toxin on the day of immunization and then two days later [43]. Along with EAE lesions resembling plaques in MS autopsies, EAE is advantageous in that its myelin-reactive CD4+ T-cell inflammation provides an ample platform for studying the T-cell inflammatory components of MS [43].

Substantial evidence exists for the involvement of macrophages/microglia in EAE. However, as mentioned in other animal models of CNS injury, there is a clear dichotomy in the roles of these phagocytic cells in EAE.

5. Detriments and Benefits of Macrophages/Microglia in EAE

The majority of studies demonstrating the detrimental or beneficial involvement of macrophages/microglia in EAE used loss-of-function approaches, in which macrophages/microglia were inhibited or depleted and the subsequent histopathology and behavioural symptoms monitored. For example, in a parabiosis study in which hematopoietic cells were ablated in the recipients but not the donor animals, it was shown that EAE progression was correlated with macrophage infiltration in recipient mice [14]. In other studies, inhibitors to macrophages or their depletion resulted in an attenuation of EAE progression [44–46]. Heppner et al. [47] used CD11b-HSVTK transgenic mice with systemic administration of ganciclovir to reduce macrophage/microglial density. In this study, it was found that ganciclovir administration corresponded to a suppression in the development of EAE, suggesting that macrophage/microglia activation is detrimental in EAE. Agrawal et al. [48] showed that ablation of both matrix metalloproteinase-2 (MMP-2) and MMP-9, which reduced leukocyte penetration across the BBB, suppressed EAE. In addition, Bartholomäus et al. [49] reported the reactivation of CNS-infiltrating pathogenic T cells by MHC class II-presenting macrophages in EAE. In another study, Nikić et al. [50] observed mitochondrial pathology and focal axonal degeneration being initiated by the macrophage-mediated production of reactive oxygen and nitrogen species (ROS and RNS). A regression in axonal degeneration was noticed when this group neutralized the ROS and RNS. In another study providing evidence for a detrimental role of microglia, Rasmussen et al. [51] found that the activation of these cells correlated with degradation of synaptic proteins as well as with atypical phosphorylation of neurofilaments in the cerebral cortex. Another study deleted galectin-1, a critical deactivator of M1 microglia activation secreted by astrocytes, and observed pronounced inflammation-induced neurodegeneration, demonstrating the detrimental effects of M1 polarized microglia [52].

In contrast to these studies reporting detrimental roles of macrophages/microglia in EAE, three studies demonstrated beneficial roles for these cells, particularly those of the M2 variety. In this regard, the transfer of M2-polarized monocytes resulted in a suppression of EAE [53, 54]. Finally, Denney et al. [55] reported that activation of invariant NKT cells increased the proportion of M2 macrophages in the CNS of EAE mice and this led to the attenuation of clinical signs.

In these studies, investigators employed gain-of-function or loss-of-function approaches to determine the roles that macrophages/microglia perform in EAE. When macrophages of the M2 subset were present, EAE severity was dampened, demonstrating a beneficial role for these cells. However, when macrophage activity was depleted, an improvement in EAE resulted, implying a detrimental role for these cells. To further study the role of macrophages/microglia in the processes of demyelination and remyelination, other animal models of MS must be used. In EAE, demyelination and remyelination are difficult to quantitate as the severity, lesion location, and temporal specifications of the pathophysiology vary from animal to animal. Models which can reproducibly induce a focal demyelinating lesion in a white matter tract would offer a significantly better approach to studying the effect that these cells have on the de-/remyelination process. In addition, the latter demyelinating models normally do not have extensive T-cell representation, allowing effects of drugs on macrophages/microglia to be determined. The reproducibility of these models allows for

the examination of the effect that loss-of-function, gain-of-function, or pharmacological treatment experiments have on the demyelination/remyelination process [56]. The following sections discuss the roles that have been elucidated for macrophages/microglia in focal demyelinating models, namely, the cuprizone and lysolecithin models.

6. The Cuprizone Demyelinating Model

The cuprizone model is the more commonly used of the two main focal demyelinating models, partly due to its ease of administration in the diet to produce injury.

Cuprizone is a copper chelator that is normally fed to mice for 4 to 6 weeks to induce significant, reproducible demyelinating lesions in the corpus callosum, hippocampus, anterior commissure, olfactory bulb, optic chiasm, brain stem, cerebellum, caudate putamen, cerebral cortex, and the cingulum [57–64]. The mechanism of demyelination is thought to be due to selective toxicity to oligodendrocytes, specifically through disruption in the mitochondrial complex IV of these myelin-forming cells. Discontinuation of cuprizone results in remyelination in the corpus callosum, usually through recruitment and differentiation of OPCs [43]. Significant M/M accumulation has been reported in lesions in this model, allowing the specific involvement of these cells in demyelination and remyelination to be examined [59, 61, 65–68]. The cuprizone model is advantageous to other focal demyelinating models in that the insult is easy to administer. However, a longer period is normally required for demyelination to occur, typically taking a few weeks. In addition, once cuprizone is removed, remyelination occurs very quickly, posing a difficulty in examining remyelination-specific events, such as the effect of remyelinating drugs on white matter tracts.

In the last fifteen years, there has been a large amount of research using this model, yielding many reports of the detrimental and beneficial roles of macrophages/microglia in de-/remyelination. The observed detriments will be first discussed followed by an overview of the beneficial aspects associated with these cells.

7. Detriments of Macrophages/Microglia in the Cuprizone Model

Wergeland et al. [69] used the cuprizone model on mice being supplemented with vitamin D3 in their diet. With high and very high doses of this vitamin, the amount of microglia activation was decreased, correlating with a reduction in white matter demyelination. Although these results suggest that activated microglia and infiltrating macrophages may be aiding the demyelination process, one cannot rule out the possibility of vitamin D3 having a protective effect on its own and thereby reducing macrophage/microglia activity. Another study found that administration of erythropoietin to mice fed cuprizone reduced vestibulomotor impairment, an observation that was associated with a decrease in microglia activation in the corpus callosum [70]. Yoshikawa et al. [71] found that pharmacological inhibition of 5-lipoxygenase (5-LO), an enzyme involved in the biosynthesis of leukotrienes, reduced cuprizone-induced axonal damage and motor deficits. Cuprizone-induced demyelination, however, was not attenuated. This observed reduction in axonal damage was associated with a decrease in microglial activation, suggesting that the 5-LO pathway contributes to microglial activation and neurotoxicity. Another study examined the expression of iNOS in the M1 proinflammatory subclass of macrophages/microglia. In that study, the authors found that the administration of fumaric acid esters, compounds which significantly inhibited LPS-induced nitric oxide production by microglia, slightly accelerated remyelination in the corpus callosum [63]. These findings suggest a negative role for iNOS in demyelinating lesions. When cuprizone-treated mice were administered the microglia inhibitor minocycline, a decrease in demyelination and an improvement in motor coordination behaviour was observed [72–74].

17 beta-estradiol (E2) is a form of estrogen that has been shown to reduce symptoms in EAE [75]. When this compound was administered to cuprizone-treated male mice, there was a reduction in demyelination accompanied by a delay in microglia activation. This correlation implies a protective effect corresponding to decreased microglia activation. Millet et al. [76] demonstrated that the injection of a proteasome inhibitor, lactacystin, into the corpus callosum during the remyelination process in cuprizone-treated mice resulted in a large improvement in remyelination, corresponding with attenuation of the recruitment of macrophages/microglia. Macrophage inflammatory protein-1α (MIP-1α) is a protein that has been associated with the recruitment of macrophages/microglia to demyelinating lesions in cuprizone treatment. A study conducted by McMahon et al. [77] found a significant decrease in demyelination in mice deficient in MIP-1α, indicating a role of macrophages/microglia in promoting demyelination.

These studies point to a harmful role for macrophages/microglia in cuprizone-induced demyelination. Decreased microglia activation corresponding with decreased demyelination, motor impairment, and increased remyelination, suggests a negative role for microglia in the demyelination/remyelination process. However, there is evidence demonstrating a beneficial role for these cells as well.

8. Benefits of Macrophages/Microglia in the Cuprizone Model

In contrast to the studies mentioned above, there are several studies that have noted beneficial roles for macrophages/microglia in cuprizone-induced demyelination. Olah et al. [78] isolated microglia from the corpus callosum of mice in a cuprizone experiment and performed a genome-wide gene expression investigating the upregulated genes associated with remyelination or demyelination. In that study, they found that microglia displayed a phenotype associated with the phagocytosis of myelin debris as well as with the recruitment of OPCs through the expression of cytokines

and chemokines. The study provided transcriptomic evidence for the ability of microglia to support remyelination. Jurevics et al. [79] found that genes related to macrophages/microglia appeared in a temporal fashion corresponding to phagocytosis of myelin debris and repair of lesions, suggesting beneficial roles of these cells in clearing the inhibitory environment for repair. Another study looked at the role astrocytes played in providing prorepair signals to microglia in a demyelinated environment. Skripuletz et al. [80] ablated astrocytes in the cuprizone model using a TK transgene under the control of the astrocyte-specific promoter, glial fibrillary acidic protein (GFAP). These mice showed a failure to remove collapsed myelin debris, which was associated with a reduction in microglial activation, suggesting the importance of astrocyte signalling to macrophages/microglia in myelin debris clearance.

The major histocompatibility complex class II (MHC II) is predominantly present on microglia to present antigens to lymphocytes crossing the BBB. In MHC II null mice, there was delay in remyelination and differentiation of OPCs, pointing to the potential beneficial role played by microglia in remyelination [81]. The same group also found that in mice lacking the microglial enzyme, iNOS, and in mice lacking the microglial cytokine, tumor necrosis factor-α (TNF-α), there was a significant delay in remyelination and more severe demyelination, indicating the importance of the macrophages/microglial expression of iNOS and TNF-α in remyelination [82, 83]. Morell et al. [84] suggested that macrophages/microglia may be important in recruiting OPCs and stimulating their differentiation into mature myelinating oligodendrocytes. This was postulated when they observed that upregulation of mRNA transcripts for myelin-associated glycoprotein (MAG) and myelin basic protein (MBP) coincided with the accumulation of macrophages/microglia, before any remyelination was observed. Mason et al. [85] induced demyelination with cuprizone in mice lacking interleukin-1β (IL-1β), a cytokine normally secreted by macrophages/microglia. In that study, they found an impairment in remyelination in the homozygous IL-1β null mice, pointing to the macrophage/microglial-derived secretion of IL-1β as a potentially beneficial molecule for remyelination.

From these studies, it is evident that the cuprizone model of demyelination has been able to provide a large body of experimental evidence concerning the beneficial roles played by macrophages/microglia in de-/remyelination. These studies have demonstrated that macrophages/microglia have the potential to clear myelin debris, promote the recruitment and differentiation of OPCs, and release cytokines which may be beneficial for the remyelination process. As to when these cells are detrimental or beneficial will most likely depend on other mediators in the microenvironment, the extent of injury, and the temporal coordination between signalling molecules from other sources such as astrocytes. The following sections will now examine the body of evidence accumulated in the other commonly used focal demyelination model, the lysolecithin injury.

9. The Lysolecithin Demyelinating Model

Lysolecithin (lysophosphatidylcholine) is a demyelinating chemical that is administered through a stereotactic injection into white matter tracts in the CNS [43]. The dorsal and ventral funiculi of the thoracic and lumbar spinal cord are the most common injection targets, although the corpus callosum is sometimes used as well. Noticeable demyelination occurs hours after injection of the chemical, with significant demyelination lasting about seven to ten days. Substantial remyelination is normally evident twenty-one days after lesion formation [86]. Considerable macrophage infiltration and activated microglia are observed in the lesions with minimal T-cell involvement [87, 88]. Imai et al. [88] found that transplanted GFP-positive, bone marrow-derived macrophages represented the major cell population in lysolecithin-induced mice at days 2, 4, and 7-time points that are early in the demyelination/remyelination process. This model is advantageous in that it is quick to induce with a prolonged remyelination period, thus allowing processes promoting or interfering with remyelination to be studied more comprehensively than with the cuprizone model.

10. Detriments of Macrophages/Microglia in the Lysolecithin Model

The lysolecithin model has provided considerable insight into the biology of macrophages/microglia in the de-/remyelination process. Although not as frequently used as the cuprizone model, due largely to its greater challenge in producing injury as it involves a stereotaxic surgery, it has significant potential in elucidating the impact of specific treatment and intervention on demyelination and remyelination. In particular, its rapid induction of demyelination as well as its drawn out course in remyelination offers significant advantages over the cuprizone model, characteristics which may draw in more researchers into employing this model in the study of remyelination.

The detriment of macrophages/microglia in the lysolecithin model was suggested by the result that corticosteroid-treated animals had an enhancement in remyelination associated with a reduction in the number of macrophages/microglia at the lesion site [89]. Another study administered progesterone to adult male mice with lysolecithin demyelination [90] and found that on day 7, there was reduced representation of macrophages/microglia whilst remyelination, and numbers of OPCs and oligodendrocytes were elevated. Furthermore, Schonberg et al. [91] activated macrophages/microglia using zymosan, a toll-like receptor-2 agonist, following lysolecithin injury and observed a loss of OPCs and oligodendrocytes; the results suggest that macrophage/microglia activation can hinder remyelination through an inhibition of OPC recruitment and differentiation.

In summary, there is good data suggesting the negative roles of macrophages/microglia in demyelination/remyelination. As demonstrated in the aforementioned studies, when macrophages and microglia are diminished, the recruitment

and differentiation of OPCs are increased, resulting in a better outcome for remyelination. This macrophage/microglia-mediated impairment of OPC recruitment and differentiation may be due to a variety of factors, including the release of toxic molecules. The next section will outline evidence for the positive roles of these cells in lysolecithin-induced demyelination.

11. Benefits of Macrophages/Microglia in the Lysolecithin Model

The benefits of macrophages/microglia in the lysolecithin model was first highlighted by Triarhou and Herndon [92, 93] who showed that depleting macrophages with silica quartz dust and dexamethasone resulted in an impairment in the clearance of collapsed myelin debris as well as a delay in the remyelination process. Using the clodronate liposome method to deplete macrophages, Kotter et al. [94] described that the depletion of macrophages soon after injury (day 1) significantly reduced remyelination 21 days after lysolecithin demyelination. When the authors administered clodronate liposomes later in the remyelination phase (day 8), the outcome of remyelination did not change, indicating that infiltrating macrophages were important in the early stages of remyelination. A later study carried out by the same group [95] found that depletion of macrophages resulted in delayed recruitment of OPCs to the lesion site; the mechanism of the requirement of macrophages was likely related to their clearance of myelin debris that otherwise was an inhibitory milieu for the differentiation of OPCs [96]. A recent study paired the circulatory systems of a GFP-expressing young mouse with a lysolecithin-induced demyelinated old mouse and found macrophages from the young mouse infiltrating the lesion in the old mouse and stimulating an increase in OPC differentiation as well as remyelination [97]. This study very clearly demonstrated the beneficial role macrophages play in promoting remyelination in the lesioned CNS as well as served to demonstrate the ability of young macrophages to enhance the recovery of an aged CNS, an environment in which remyelination and macrophage/microglia accumulation were significantly delayed. In support of the benefits of macrophages/microglia, the early administration of minocycline to demyelinated rodents inhibited macrophage/microglia activation examined at 1 and 3 days postlesion and reduced oligodendrocyte repopulation and remyelination [98].

This model of demyelination has accumulated a significant amount of knowledge demonstrating the benefits and detriments of macrophages/microglia in remyelination. While there is data suggesting that these cells may be inhibiting the recruitment of OPCs to the lesion, there is evidence suggesting the alternative. The recurrent observation of these cells being critical in the clearance of inhibitory myelin debris seems to be a requisite for the migration and subsequent differentiation of OPCs into the lesion. Certainly, the temporal coordination of cytokines and signalling molecules, the size and extent of the lesion, and the inhibitory properties of the extracellular environment likely dictate whether these cells will be beneficial or detrimental to the remyelination process. More studies using this model will inevitably yield more information on the complex roles characterizing these cells, thus allowing therapies to be developed which take advantage of the beneficial aspects and suppress the detrimental aspects.

12. Basis for Detriments of Macrophages/Microglia

There is a large accumulation of evidence demonstrating the harmful aspects of macrophages/microglia in the demyelinated lesion. Mechanisms underlying these detrimental roles may include the recruitment and reactivation of T cells in the CNS through the release of proteases, the production of proinflammatory cytokines, and the release of reactive oxygen species to induce neurotoxicity and OPC toxicity through excitatory amino acids. This section will discuss some of the evidence supporting these possible mechanisms.

In order for pathogenic T cells to enter the CNS parenchyma, they must be able to cross the parenchymal basement membrane. This is a laminin-containing basement membrane which lies in direct apposition to the CNS parenchyma and venules within the CNS. For these cells to successfully transmigrate this membrane, proteases such as the MMPs must be present. Macrophages/microglia are a major source of MMPs, and when selective MMPs are blocked, the amelioration of EAE clinical signs is observed [48, 99–101]. These observations suggest that the secretion of MMPs by macrophages/microglia aid in the recruitment of pathogenic T cells to the CNS.

Macrophages/microglia also assist in the reactivation of T cells once they enter the CNS parenchyma. In order for a T cell to contribute to CNS pathogenesis, it must be reactivated with antigen presenting cells within the CNS parenchyma. It has been observed that macrophages/microglia upregulate MHC II molecules when they are activated. MHC II molecules are required for antigen presentation to T cells [102]. These observations suggest the capability of activated macrophages/microglia to reactivate primed T cells entering the CNS. It should also be noted that the expression of MHC II has been correlated with the infiltration of T cells as well as with the progression of EAE [103].

In addition to recruitment and reactivating T cells, macrophages/microglia have also been observed to strip myelin, as well as kill neurons and OPCs [104–106]. One of the mechanisms explaining this macrophage/microglia-mediated toxicity is through the production of cytokines, glutamate, and reactive oxygen species [27]. When macrophages/microglia are activated, they release an array of inflammatory cytokines, such as TNF-α and IFN-γ. These cytokines induce the release of glutamate. Excessive glutamate stimulation on N-methyl-D-aspartate (NMDA) receptors results in mitochondrial death and ultimately excitotoxic neuronal and oligodendrocyte death [106]. When glutamate release is blocked, EAE progression is attenuated, providing evidence for glutamate excitotoxicity as a plausible mechanism for macrophage/microglia-mediated toxicity [107]. In addition to stimulating the release of glutamate, proinflammatory cytokines and chemokines promote inflammation and antigen presentation, thereby mediating the recruitment and reactivation of T cells

to the lesion [108]. Finally, the release of free radicals, such as nitric oxide (NO), has been shown to induce oxidative damage to neurons and oligodendrocyte precursor cells [109].

It is evident that the array of detriments associated with macrophage/microglial activity in MS and its animal models function through many mechanisms of action. Such modes of action include the expression of proteases to aid in the recruitment of T cells into the CNS, the upregulation of molecules associated with antigen presentation, thereby aiding in the reactivation of pathogenic T cells, and through the release of inflammatory cytokines, free radicals, and glutamate. Developing therapies which target these mechanisms will certainly offer hope in the quest for improving the outcome of remyelination.

13. Basis for Benefits of Macrophages/Microglia

From the array of studies conducted with the different animal models of MS, it is clear that macrophages/microglia, although being shown to be predominantly detrimental, have a variety of beneficial effects. The mechanisms of these beneficial effects are thought to be due to the production of growth factors, the removal of inhibitory debris and toxic products, and through the removal of inhibitory extracellular matrix (ECM) molecules. This section entails a discussion of some of the evidence supporting these plausible mechanisms.

Macrophages/microglia have been reported to secrete many well-known neurotrophic factors, which may help in the recruitment and differentiation of OPCs, as well as in the regeneration of axons. Herx et al. [110] showed that the production of IL-1β by microglia regulated the production of ciliary neurotrophic factor (CNTF), an important growth factor for the survival of oligodendrocytes. Studies have also demonstrated the production by macrophages/microglia of other neurotrophic factors, such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophin-3 (NT-3) [111, 112]. As mentioned above, Yin et al. [29] reported that axonal regeneration after optic nerve injury was partially due to oncomodulin, a growth factor secreted by macrophages. Overall, there is evidence to support the claim that macrophages/microglia promote remyelination and axonal regeneration through the secretion of beneficial growth factors. A challenge remains, though, in determining the temporal and spatial characteristics in which these benefits are most likely to occur.

Another beneficial mechanism of macrophages/microglia may be through the removal of inhibitory debris or toxic products. As mentioned previously, Kotter et al. [95] found that macrophages were important for the clearance of myelin debris and in enabling subsequent remyelination to occur. These authors found in a later study that myelin debris acted to inhibit OPC differentiation into mature, myelinating oligodendrocytes [96], thus supporting the ability of macrophages/microglia to clear debris as a beneficial role in remyelination. In another study, the depletion of macrophages with silica dust resulted in a hindered clearance of myelin debris and significantly delayed remyelination [92]. In an Alzheimer's-like disease model, it was found that macrophages recruited to the CNS cleared intracerebral Aβ deposits [38]. Altogether, it can be seen that there is evidence supporting the capability of macrophages/microglia in clearing inhibitory myelin debris as well as toxic products such as Aβ deposits, ultimately associating with a decrease in the severity of the disease.

Finally, our group has shown that macrophages/microglia act beneficially to remove inhibitory extracellular matrix molecules. As mentioned previously, CSPGs are normally deposited around a CNS lesion. We have shown that NG2, a type of proteoglycan, hinders the differentiation of OPCs [113]. It was found that the production of MMP-9 by macrophages/microglia cleared the accumulation of NG2 within a demyelinating lesion, leading to subsequent oligodendrocyte maturation and remyelination.

Although the mechanisms discussed above serve to explain some of the benefits observed with macrophages/microglia, there are likely other positive mechanisms that aid in the remyelination process. One such mechanism may be through the stimulation of astrocytes to secrete trophic factors as well as to reseal the blood-brain barrier (BBB) [114]. By determining the mechanisms involved in the beneficial aspects of these immune cells, we will be able to better target these endogenous processes with therapies that may stimulate remyelination and axon regeneration. This can already be seen by some of the therapies already in use for the treatment of multiple sclerosis. The next section will discuss some of these therapies and briefly describe the mechanisms in which the macrophage/microglia population is targeted.

14. Therapies Involving Macrophage/Microglia Activity

There are currently seven therapies approved by the US Food and Drug Administration (FDA) for treatment of multiple sclerosis. All of these therapies function by modulating the immune system, with many having broad effects affecting multiple components of both innate and adaptive immunity. While T cells are most commonly targeted by these medications, there are reports describing the involvement of macrophages/microglia in these therapies. This section will briefly describe five of the seven licensed therapies in which significant involvement of macrophages/microglia has been documented.

Glatiramer acetate (Copaxone) is a first-line therapy that has been shown to reduce the relapse rate and progression of disability in patients with relapsing remitting MS [115]. This compound is a random polypeptide composed of four amino acids designed to mimic myelin basic protein (MBP), a critical component of the myelin sheath [116]. The major mechanism of action is to induce T cells of the T helper type 2 subset (Th2). These cells secrete the anti-inflammatory cytokines interleukin-4 (IL-4), IL-5, IL-6, IL-10, and IL-13 and transforming growth factor-β (TGF-β). In addition to the induction of Th2 cells, glatiramer acetate has been shown to have an effect of macrophages/microglia, with many studies suggesting a role in promoting an anti-inflammatory M2 phenotype. It was found that glatiramer acetate increased

the expression of the anti-inflammatory cytokine IL-10 and reduced the expression of the proinflammatory cytokine tumor necrosis factor-α (TNF-α) in macrophages/microglia [117–119]. Another study found that this compound inhibited the production of NO, suggesting the polarization away from an M1 phenotype [120]. Finally, this compound has been demonstrated to increase the phagocytic activity of microglia and monocytes, possibly contributing to increased myelin debris clearance [121]. Altogether, it appears as though glatiramer acetate has many effects on the macrophage/microglia population, while at the same time polarizing the T-cell response to one of an anti-inflammatory nature.

Interferon-β (Avonex, Betaseron, Extavia, and Rebif) is another first line therapy which has demonstrated success in reducing the rate and severity of relapses, as well in slowing the progression of disability [121]. The main mechanisms of action seem to be in the inhibition of T-cell recruitment and activation, as well as in the modulation of cytokines [116, 122, 123]. Though the reduction in the T-cell response is quite evident, aberrations in the macrophage/microglia response may also contribute. For instance, interferon-β has been demonstrated to reduce the proliferation of both macrophages and monocytes as well as reduce the expression of MHC II on these cells [124]. This reduced expression of MHC II may be a reason for the decreased activation of T cells, as the antigen-presentation capabilities of macrophages/microglia would be hindered. Another example of macrophage/microglia involvement is demonstrated in the study by Hall et al. [124], which highlighted the reduction of toxic microglia-derived respiratory bursts as a result of interferon-β interaction. As interferon-β is a cytokine, many other unknown mechanisms may be occurring, possibly involving other aspects of the macrophage/microglia response.

Fingolimod (Gilenya) has demonstrated success in reducing the relapse rate as well as the rate of disability progression [116, 125]. It acts by modulating the sphingosine 1-phosphate receptors, thereby preventing lymphocyte recruitment, as these receptors are required for the exit of lymphocytes from secondary lymphoid organs to the CNS [126]. In addition, fingolimod induces an anti-inflammatory phenotype in activated macrophages. This is supported by the observation of decreased production of proinflammatory cytokines and free radicals by macrophages upon application of this drug [127].

Mitoxantrone (Novatrone) is a cytotoxic agent that functions through the intercalation of DNA [128]. Due to this broad mechanism of action, it affects many of the different types of immune cells, resulting in widespread immunosuppressive activity [129]. This immunosuppression results in the inhibition of monocyte and lymphocyte migration into the CNS [130]. Reductions in the proinflammatory cytokines, TNF-α, IL-2, and IFN-γ, have also been observed [131]. This reduction in monocyte activity may explain an observation made in which macrophage-mediated myelin degradation was inhibited [132].

Dimethyl fumarate (Tecfidera) is a recently approved first-line therapy for multiple sclerosis. It is an oral tablet thought to act by decreasing the expression of NF-κB dependent genes [133, 134]. These genes regulate the expression of inflammatory cytokines, and as such, studies have reported decreased production of TNF-α, IL-1β, IL-6, and NO in microglia [135]. Adding to these effects, when dimethyl fumarate is administered to mice with EAE, a significant reduction in infiltrating macrophages is observed in the lesions [136]. As is the case with the aforementioned medications, there may be many modes of action that are still unknown. Nonetheless, involvement of macrophage/microglia seems to be consistent across the approved medications used for multiple sclerosis.

15. Conclusion

Macrophages/microglia are implicated in promoting demyelination; yet remyelination in MS lesions appears to require these cells as repair occurs in the presence of macrophages/microglia [137, 138]. From the research conducted on macrophages/microglia in models of CNS injury, including EAE, cuprizone-, and lysolecithin-induced demyelination, it is evident that a complex dichotomy exists in the roles of these cells in the demyelination/remyelination process. More detailed studies analyzing the spectrum of activities of macrophages/microglia at different time points in the focal demyelination models, as well as studies depleting and activating these cells at different temporal points will only serve to dissect the dichotomy presented to us. A thorough understanding of when macrophages/microglia are beneficial or detrimental in the demyelination/remyelination process will allow us to develop therapeutic interventions which take advantage of the respective characteristics, with the ultimate goal to enhance remyelination and suppress demyelination. In addition to the importance of the cuprizone and lysolecithin demyelination models in being critical tools for dissecting the dichotomy of macrophages/microglia, studies using EAE will serve to complement what we learn from the focal demyelinating models, altogether serving to further our understanding of the complex processes of de-/remyelination in injuries to the CNS.

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Review Article

Role of Microglia in CNS Autoimmunity

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Multiple sclerosis (MS) is the most common autoimmune disease of the central nervous system (CNS) in the Western world. The disease is characterized histologically by the infiltration of encephalitogenic T_H1/T_H17-polarized $CD4^+$ T cells, B cells, and a plethora of myeloid cells, resulting in severe demyelination ultimately leading to a degeneration of neuronal structures. These pathological processes are substantially modulated by microglia, the resident immune competent cells of the CNS. In this overview, we summarize the current knowledge regarding the highly diverse and complex function of microglia during CNS autoimmunity in either promoting tissue injury or tissue repair. Hence, understanding microglia involvement in MS offers new exciting paths for therapeutic intervention.

1. Multiple Sclerosis: The Most Frequent Autoimmune Disease of the CNS

An autoimmune disease is characterized by the loss of self-tolerance of the immune system, which can be caused by either genetic or environmental factors or a combination of both [1]. As a consequence of this malfunction, an immune response is initiated against certain cell types or even entire organs of the body. For the central nervous system (CNS) several autoimmune diseases are described of which multiple sclerosis (MS) is the most common form, affecting approximately 2.5 million people worldwide, mainly in the third and fourth decades of live. While the exact etiology of MS is still unknown, much progress has been made in understanding its pathology. MS comprises a blood-brain-barrier (BBB) disruption accompanied by an activation of macrophages/microglia as well as T- and B-cell infiltration into the CNS, ultimately resulting in demyelination and degeneration of neuronal structures [2]. MS can be clinically divided into different forms. Most patients experience relapsing-remitting stage (RRMS) of the disease, which in many cases results in continuous disease progression called secondary progressive MS (SPMS). On the other hand, some patients suffer from primarily progressive MS (PPMS), characterized by a continuously progressing disease course [2]. To date no cure for any form of MS exists, but several treatment options which might reduce the symptoms are available [3]. One of these approaches compromises the application of IFN-β, which is thought to be anti-inflammatory in RRMS and thereby reduces the relapse rate [3–5]. Unfortunately, even though IFN-β is well tolerated by patients, approximately 50% of them respond to and benefit from the treatment [5]. The effects of IFN-β are complex and far from being fully understood. Profound insights into the pathogenic mechanisms involved in MS as well as possible therapeutic interventions were gained through the use of experimental autoimmune encephalitis (EAE), the most used animal model for CNS autoimmunity [6, 7]. Several key features of MS, such as paralysis, weight loss, demyelination, and inflammation, observed in human patients, are recapitulated during EAE in rodents [7]. Depending on the strain, EAE can be induced by active immunization with myelin derived proteins such as myelin oligodendrocyte glycoprotein (MOG), myelin basic protein (MBP), or proteolipid protein (PLP) in combination with an adjuvant, usually complete Freund's adjuvant (CFA) [7]. CFA contains inactivated mycobacteria and is thought to break peripheral tolerance, which results in the induction of CNS autoimmunity. CFA is recognized by pattern recognition receptors such as Toll-like receptors (TLRs). Especially, myeloid differentiation primary response gene (88) (MyD88),

TLR7, and TLR9 have been found to be essential disease modifiers. In addition to these surface and endosomal receptors, newly discovered endosomal molecules such as retinoic acid inducible gene- (RIG-) I and melanoma differentiation-associated protein- (MDA-) 5 also have been shown to be crucial for EAE induction [8, 9]. Some of these disease modifying recognition receptors release type I interferons (IFNs) upon activation that in turn robustly change both the innate and adaptive arms of autoimmunity in mice [9–12]. However, for C57BL/6 mice the EAE model induced by MOG_{35-55} peptide is thought to be a monophasic chronically active disease without significant recovery and relapse phases, thereby only partially reflecting the clinical course found in MS patients [13]. Nevertheless, this EAE model in addition to the increasing availability of (cell type-)specific knock-out mice has greatly expanded our understanding of MS pathology and might open new avenues for specific treatment options in the future.

2. Microglia-Resident Macrophages of the CNS

Microglia cells are resident tissue macrophages located in the CNS and are considered to be a patrolling immune competent cell type within the parenchyma [14–17]. Microglia make up around 10% of the cells in CNS and are evenly distributed in the parenchyma of a healthy brain [14]. In contrast to neurons and macroglia (oligodendrocytes and astrocytes), microglia originate from the primitive hematopoiesis within the yolk sac (YS) and migrate to the neuronal tube during embryogenesis [17, 18]. Recently, we could identify lin^--erythromyeloid precursors as the genuine microglia progenitors on a stem cell level [19]. These pioneer cells continue their physiological and morphological development on their way from the YS to the developing brain, where the mature cells finally reside and build the ultimate pool of microglia [17, 19, 20]. By using parabiotic mice, a contribution of bone marrow-derived phagocytes (BMDPs) to the pool of existing microglia throughout life could be ruled out [20–22]. In fact, BMDP engraftment from the circulation can only be achieved after significant "priming" of the host CNS, for example, by irradiation-induced changes of the BBB or alterations of the tissue micromilieu [19, 23–25].

Due to their morphology, microglia cells are ascribed to be in a "resting" state under healthy conditions. This term is somewhat misleading, since *in vivo* imaging has revealed that microglia actively scavenge and monitor with their ramified branches the environment of the CNS for pathogens [26, 27]. Upon tissue damage, or inflammation, viral or bacterial insult, microglia change their morphology towards an amoeboid shape by retracting these branches. In addition to the morphological differentiation, several surface markers, such as F4/80 or Mac-1, which is typical for macrophages, are upregulated [28, 29]. The status of activation can be further subdivided into "classically" (M1) and "alternatively" (M2) activated microglia [15, 30] (Figure 1). This subdivision is based on function that while M1 microglia are often associated with acute infection, M2 cells play a role in tissue remodelling, repair, and healing [30]. However, M1 macrophages are also vital and important for the defence against microorganisms. The functional classification relies on the microenvironment of chemo- or cytokines as a result of microbial products or damaged cells [30]. IFN-γ and lipopolysaccharide (LPS) polarize microglia towards the M1 state and induce the release or expression of interleukin- (IL-) 1, IL-6, IL-12, IL-23, and inducible nitric oxide synthase (iNOS). On the other hand, the presence of IL-4, IL-10, and IL-13 turns microglia into M2 cells, which produce IL-10 and express arginase 1 [30, 31]. However, one has to keep in mind that this distinction into M1/M2 is a simplification and represents the extreme states. During disease both of these extremes as well as intermediate states may be present. However, the described subdivision of M1 and M2 cells nicely reflects the Janus-like behavior of microglia regarding their promotion of either tissue injury or repair. However, more and more evidence has accumulated that microglia do not only take part in immunological processes but also play a role in non-pathological conditions by, for example, eliminating or remodelling synapses and support of myelin turnover [32–34]. Among all the described functions of microglia, this review concentrates specifically on the involvement of microglia in autoimmune diseases of the CNS. In particular, we elucidate the mechanisms of microglia activation and the subsequent results of activation. Finally, we highlight current ideas to interfere in microglia activation as promising therapeutic strategies for MS.

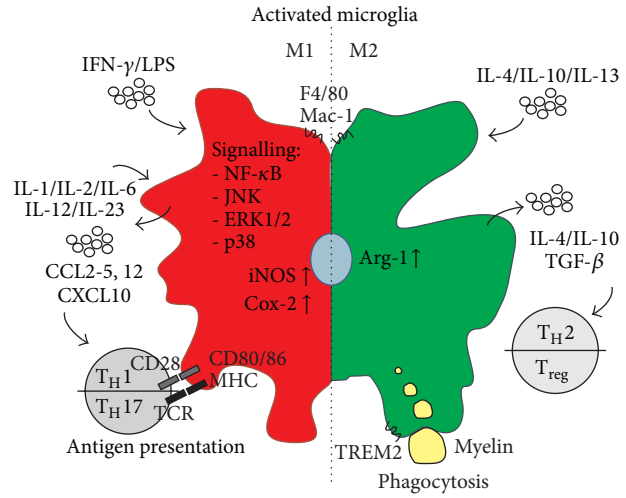

Figure 1: Polarization and function of activated microglia in CNS autoimmune inflammation. Microglia are activated by diverse stimuli, which define the polarization status of the cell. While IFN-γ/LPS promote the proinflammatory M1 status, IL-4/IL-10 or IL-13 induce the anti-inflammatory M2 status. M1 microglia take part in the attraction and differentiation of pathogenic T_H1/T_H17 T-cells, whereas M2 microglia promote phagocytosis of myelin debris, which is important for remyelination.

3. Function of Microglia in CNS Autoimmunity

3.1. Activation of Microglia as Hallmark of Disease. For several decades, the activation of microglia has been described in the damaged CNS during the pathology of MS/EAE, reflecting an initial event in MS pathology [35]. Even in early stages of MS, activated microglia clusters, so-called microglia nodules, are found in preactive lesions in the white matter of MS patients [36, 37]. In response of becoming activated, microglia also start to proliferate during MOG_{35-55}-induced EAE [22]. In order to gain further insights into the involvement of activated microglia, a CD11b-*HSVTK* transgenic mouse line was generated [38]. In this mouse model the *HSVTK* gene is driven by the CD11b promoter, which is only expressed in cells with myeloid origin including microglia and macrophages. Importantly, *HSVTK* acts suicidal upon ganciclovir treatment. Thus, CD11b-*HSVTK* represents a pharmacogenetically inducible *in vivo* model of microglia depletion. MOG immunization of ganciclovir-treated CD11b-*HSVTK* mice significantly repressed disease onset and the severity of clinical EAE signs. The importance of microglia activation during EAE pathology was confirmed by application of macrophage inhibitory factor (TKP) or minocycline, which also attenuated EAE symptoms [39, 40]. Recently, the involvement of the complement system, which usually plays a part in defence and elimination of microorganisms in the adaptive immune response [41], in microglia activation in EAE was shown [42]. Under pathological conditions, like MS, a first disease-causing stimulus primes microglia, which upon a second stimulus result in overactivated microglia [42]. The presence of primed microglia was observed in MS lesions and in a knock-out of complement receptor-1-related protein y (Crry) [42]. During EAE primed microglia led to overactivated microglia resulting in an enhanced clinical severity in Crry-deficient mice [42]. Thus, microglia activation presents a histological hallmark in MS pathogenesis. However, during the disease, activated microglia might fill two positions depending on their polarization, M1 cells are involved in the pathogenic T-cell response (T_H1 and T_H17), while M2 microglia are designated as being protective in MS [43, 44]. Therefore, it is important to understand the processes and mechanisms of microglia activation and polarization.

Recently, miRNA124 (miR-124) was identified to be a key regulator and modulator of microglia/monocyte activation [29]. miR-124, which is highly expressed in ramified microglia, belongs to the family of noncoding microRNAs, which are known to have regulatory functions such as promoting mRNA degeneration or interfering with mRNA translation [29, 45]. Interestingly, while during onset and peak of EAE expression of the surface markers CD45 and MHC class II was upregulated in microglia, the expression of miR-124 was repressed [29]. miR-124 targets C/EBP-α and thereby decreases the expression of C/EBP-α and indirectly Pu.1, both important myeloid regulatory transcription factors responsible for expression of activation markers [29]. Finally, intravenous treatment of miR-124 in the preclinical phase as well as after onset of the disease inhibited or substantially ameliorated the clinical course of EAE. Importantly, application of miR-124 turns microglia towards an M2 polarization.

In addition, suppressor of cytokine signalling (SOCS-) 3 was identified as another key player of microglia activation [31]. SOCS proteins are known to interfere with JAK/STAT signalling. SOCS3 in particular inhibits IL-6 signalling by limiting STAT3 activation [46]. Induction of EAE in the absence of SOCS3 on myeloid cells, by using LysMCre-$SOCS3^{fl/fl}$ mice, resulted in an early onset and more severe disease course, with an overly activated STAT3/4 signalling [31]. Myeloid-specific SOCS3 deficiency polarized the microglia/monocytes towards the M1 state and induced neuronal death. Thus, SOCS3 limits activation of macrophages/microglia and controls their polarization status. Taken together, both, miR-124 as well as SOCS3, are important factors critical for microglia activation. The hereby activated microglia then take over several responsibilities depending on their polarization.

3.2. Microglia as a Source of Cytokines during Autoimmune Inflammation. Cytokines and chemokines are small secreted signalling molecules important for inter- and intracellular communication during inflammation. While both are mostly expressed at very low, basal levels in healthy conditions, the expression and secretion, primarily by microglia but also by astrocytes, are markedly increased upon CNS insult [47–50]. Microglial secretion of diverse cytokines can modulate cells in a paracrine manner but can also affect microglia in an autocrine fashion by either positive or negative feedback loops [47, 50]. Finally, the amount and composition of cytokines and chemokines in the microenvironment determines the function of microglia as indicated earlier in description of M1/M2 microglia polarization.

IFN-γ, tumour necrosis factor- (TNF-) α, IL-1β, and IL-6 are potent proinflammatory cytokines activating microglia and shifting them towards the cytotoxic M1 phenotype, thereby potentiating the inflammatory response [49–52] (Figure 1). However, to induce the complete cytotoxic M1 state, which leads to the expression of TNF-α, IL-1β, IL-2, IL-6, and IL-12, as well as iNOS and cyclooxygenase-2 (COX-2), binding of more than one cytokine is necessary [50]. Successful stimulation then triggers the expression of target genes via various signalling cascades including NF-κB, JNK, ERK1/2, and p38 [50, 53, 54]. Subsequently, constitutive activation of, for example, the NF-κB signalling cascade in microglia/macrophages in LysMCreIκB$\alpha^{fl/fl}$ mice worsens clinical symptoms of EAE and increases the expression of, for example, IL-1β and IL-6 [55]. Interestingly, NF-κB signalling is not only important in microglia, but also plays an important role in neuroectodermal-derived astrocytes during autoimmune inflammation of the CNS [56, 57].

The roles of the proinflammatory cytokines IL-12, IL-23, and IL-17 produced by classically activated M1 microglia are more sophisticated [58–60] (Figure 1). Interestingly, IL-12 and IL-23 belong to the IL-12 family of cytokines and share a similar β-chain p40 but differ in their α-chain, which is p19 for IL-23 and p35 for IL-12 [60]. Their contribution to the pathogenesis of EAE lies in the regulation, proliferation, and differentiation of naïve $CD4^+$ T cells. While IL-12 facilitates

T_H1 effector cell differentiation, IL-23 is critical for stable IL-17 expression which is ultimately important for the differentiation of pathogenic T_H17 cells [50, 60, 61]. Although T_H1 and T_H17 both hold central and distinct roles in the development and pathogenesis in CNS autoimmune inflammation, their exact functions remain to be fully resolved [61]. In contrast, alternatively activated M2 microglia express and release the cytokines IL-4 and IL-10, as well as transforming growth factor- (TGF-) β through which the differentiation of anti-inflammatory and protective T_H2 and T regulatory (T_{reg}) cells is induced [61].

In addition to cytokines, microglia are also activated by and are able to release diverse chemoattractant chemokines, such as CCL2, CCL3, CCL4, CCL5, CXCL10, and/or CCL12 [47, 48, 52, 62, 63] (Figure 1). The importance of CCL2, also known as monocyte chemoattractant protein- (MCP-) 1, and its receptor CCR2 has been investigated for various CNS pathologies such as neurodegeneration [23] and autoimmune inflammation [63–65]. Many different proinflammatory stimuli, such as IFN-γ, TNF-α, and IL-1β, can induce the expression of CCL2 in the brain [63]. Until now, two major functions have been allocated to CCL2 during CNS autoimmunity. First, it participates in the disruption of blood brain barrier integrity. Secondly it is involved in the recruitment of CCR2+CD11b+Ly-$6C^{hi}$ mononuclear leukocytes into the CNS [63, 64, 66]. Consequently, deletion or blockade of CCL2 synthesis by the chemical inhibitors significantly reduced the clinical symptoms of EAE [67–70]. In addition, therapeutic interference with the function of CCL5/Rantes reduced leukocyte activation and trafficking to the CNS [68]. In summary, by arranging the cyto-/chemokine milieu, microglia play a key role both in regulating and recruiting leukocytes into the CNS as well as macrophage polarization during autoimmune inflammation [71].

3.3. Phagocytosis Mediated by Microglia: Pathfinding for Reengineering. Phagocytosis can be seen as a double-edged sword; on the one hand, it is beneficial by clearing cellular debris, but on other the hand it can also be destructive by inducing an oxidative burst [72]. Microglia, as macrophages, are per definition phagocytes of either invading pathogens or dead cells in the CNS [72, 73]. During EAE, activated microglia are known to phagocytize myelin debris and oligodendrocytes in lesions, which in turn leads to the release of proinflammatory cytokines [71, 73] (Figure 1). One of the key receptors involved in this process during EAE is the triggering receptor expressed on myeloid cells (TREM-) 2 together with its associated signalling molecule DNAX-activating protein- (DAP-) 12, both expressed in microglia and macrophages [74, 75]. Virus-mediated overexpression of TREM2 in myeloid cells increased the clearance of myelin debris, thereby improving the tissue regeneration and consequently reducing the severity of clinical symptoms [75]. In addition to TREM2, several other receptors are important for myelin debris phagocytosis, such as complement receptor (CR) 3, signal regulatory protein (SIRP)-α, or Fcγ receptor [72].

Interestingly, remaining myelin debris inhibits the regenerative remyelination, indicated by a loss of *PDGFR*α+ oligodendrocyte precursor recruitment [76, 77]. A detailed analysis, making use of gene arrays, could determine a "single remyelination-supportive microglia" phenotype, which gradually develops [78]. Additionally, during remyelination microglia express several genes, which are known to promote oligodendrocyte differentiation [78]. Thus, efficient resolution of inflammation, by phagocytosis of myelin debris, is important for tissue reengineering.

3.4. Antigen Presentation and T Cell Priming by Microglia. MS/EAE pathology is characterized by harmful T cell infiltration [5]. For T cell activation by an antigen presenting cell (APC), two signals are prerequisites: first, antigen peptide presentation at the cell surface via major histocompatibility complex (MHC) to the specific T cell receptor [79, 80] and, second, a costimulatory interaction of CD80/86 or CD40 located on APCs with CD28 or CD40L present on T cells [71, 81]. Typically, dendritic cells represent the most prominent cell type for antigen presentation [82]. However, in addition to T cell attraction, by expressing CCL2 and CCL5, microglia can also serve as APCs by presenting myelin [83]. During MS/EAE pathology, microglia highly express MHC class I and II proteins as well as CD40 and CD80/86 [48, 84] (Figure 1). Microglia "reprime" or reactivate T cells in lesion sites and thereby exacerbate the disease by epitope spreading [71, 85, 86]. Following the T cell-APC interaction, differentiation into mature proinflammatory encephalitogenic T cells (T_H17, T_H1) or anti-inflammatory T cells (T_H2 or T_{reg}) takes place, depending on the cytokine environment [71].

3.5. Microglia as Therapeutic Targets. With the increased understanding of microglia function in MS/EAE pathology gained thus far, it might be possible to find and establish efficient therapies by targeting microglial responses. Since M1 microglia seem to be harmful during CNS autoimmune inflammation, most therapeutic ideas aim to either deactivate M1 microglia or turn them into M2 cells. Recently, galectin- (gal-) 1 was identified as a potent microglia regulator [87]. Gal-1 preferentially binds to M1 microglia, thereby modulating M1 key features, such as CCL2 and iNOS expression, by controlling NF-κB and p38 signalling [87]. Finally, gal-1 application ameliorated disease course of EAE, making it a potentially attractive therapeutic option [87]. Similar approaches were employed using tuftsin, a naturally occurring tetrapeptide known to induce phagocytosis, or ghrelin, which acts as an anti-inflammatory hormone [88, 89]. Treatment with tuftsin or ghrelin shifted microglia into M2 polarization *in vitro* and reduced EAE severity *in vivo* [88, 89]. In addition, application of compound A, a plant-derived phenyl aziridine precursor, ameliorated the clinical course of EAE, and subsequent *in vitro* analyses revealed an inhibition of NF-κB signalling by compound A in microglia [90]. β-Lapachone treatment, a natural substance from the bark of the lapacho tree, significantly reduced the expression of IL-12 family cytokines in microglia and thereby suppressed clinical severity [91].

During the termination of the acute inflammatory reaction, microglia become deactivated [15]. Therefore, the deactivation of microglia also presents a promising therapeutic concept. Several molecules, including chemokines and steroid hormones, are known as ligands regulating microglia activation status and mediate signalling via different nuclear receptors [15]. In this respect, stimulation of the oestrogen receptor- (ER-) β with synthetic ligands efficiently turned down microglia activation and thereby reduced EAE symptoms [92, 93].

Recently, the use of reprogrammed embryonic stem (ES) cell-derived microglia-like cells (ESdM) for MS was suggested [94]. Intravenously applied ESdM populate lesion sites in the spinal cord of EAE mice but do not influence the clinical course [94]. Application of lentiviral transduced ESdM with NT-3, a neurotrophic factor, significantly reduced clinical disease severity as well as the degree of demyelination and axonal damage [94].

4. Conclusion

Activation of microglia is a hallmark of MS/EAE pathology. During recent years more and more knowledge about how and why microglia become activated has accumulated. Subsequently, several investigations have already proven the feasibility of beneficially modulating microglia function in animal models of MS. Nevertheless, this path has to be taken further. There is a need for comprehensive and detailed analysis to further illuminate the triggering and signalling mechanisms of microglia in inflammation. Thoroughly understanding microglia action in the context of a disease will help us identify new targets for therapeutic approaches, which may ultimately be translated to the clinics.

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Research Article

Different Peripheral Tissue Injury Induces Differential Phenotypic Changes of Spinal Activated Microglia

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The purpose of this study is to investigate the possible different cellular marker expression associated with spinal cord microglial activation in different pain models. Immunohistochemistry and western blotting analysis of CD45, CD68, and MHC class I antigen as well as CD11b and Iba-1 in the spinal cord were quantitatively compared among widely used three pain animal models, complete Freund's adjuvant (CFA) injection, formalin injection, and chronic constriction injury (CCI) models. The results showed that significant upregulated expressions of CD45 and MHC class I antigen in spinal microglia as well as morphological changes with increased staining with CD11b and Iba-1 were seen in CCI and formalin models and not found in CFA-induced inflammatory pain model. CD68 expression was only detected in CCI model. Our findings suggested that different peripheral tissue injuries produced differential phenotypic changes associated with spinal microglial activation; peripheral nerve injury might induce spinal microglia to acquire these immunomolecular phenotypic changes.

1. Introduction

Studies in recent decades implicate that not only neurons but also glial cells (microglia and astrocytes) play important roles in the generation and maintenance of pathologic pain [1–6]. Both microglia and astrocytes are activated in the spinal cord in almost all animal models of pain, including nerve injury, traumatic injury, inflammatory, and bone cancer pain models [7–16]. However, the molecular mechanisms by which glial cells are activated and contribute to the pain situation are not fully understood. Microglial cells are the resident macrophages and the local immune cells in the central nervous system (CNS). Immune responses particularly through the activity of microglia in the spinal cord contribute to pain behaviors after injury to the nervous system [17]. Intrathecal treatment with low-dose methotrexate (an immunosuppressive drug) reduces microglial activation and attenuates pain-like behavior after nerve injury [18, 19].

Microglia are quickly activated following peripheral nerve injury or tissue inflammation [20, 21] and begin to upregulate cell surface molecules and express cytokines, chemokines, and effector molecules. Microglia are highly dynamic and undergo different cellular remodeling throughout the progression of diseases and in distinct pathological conditions [22]. In the normal CNS, microglia are characterized by ramified processes arising from a relatively small cell body, with a weak expression of molecules normally expressed by other hematopoietic lineages. It is well known that certain immune molecules, including CD11b/c, CD45, CD40, CD86, major histocompatibility complex (MHC) class II, which are markers of both macrophage and dendritic lineage cells [23], as well as Toll-like receptor, Fc receptor, and cytokine receptors [24–27], are significantly upregulated on activated microglia in a variety of experimental and clinical CNS diseases. Little is known about the expression of these immune molecules in activated microglia in different pain models [28–30]. We previously demonstrated upregulated expression of CD45 and MHC class I antigens in activated microglia along with significant morphological changes starting on day 1–3 and peaking on day 7 after peripheral formalin

injection [28]. The expression of MHC class II, CD11c, and the Fc receptor was maintained at a low basal level and did not change after the stimuli.

To understand the way activated microglia contribute to the pathogenesis of different induced pain, we compared three widely used pain models comprising injection of complete Freund's adjuvant (CFA) into the hind paw which is a predominantly inflammatory pain model, subcutaneous injection of dilute formalin into the hind paw which is a mixed inflammatory and nerve injury model, and the chronic constriction nerve injury (CCI) model which is a neuropathic pain model. The difference on microglial morphological changes between formalin and CFA model has already been detected in our previous study [31]. The aim of the present study is to address the expression level of immune molecules CD45, CD68, and MHC class I, as well as microglial markers CD11b and Iba-1, using both immunofluorescence and western blot.

2. Materials and Methods

2.1. Animals Models. Adult male Sprague-Dawley rats weighing 200–225 g (Vital River Laboratory Animal Technology Co., Ltd., Beijing) were used. All rats were housed at a temperature of 23°C (±1°C) on a 12-hour light/dark cycle with free access to food and water. The experimental protocols were approved by the Animal Care and Use Committee of Peking University Health Science Center. The care and use of animals conformed to applicable national/international guidelines.

Fifty-six rats were randomly assigned to four groups (8 rats per group). CCI rats were produced by the loose ligation of the common sciatic nerve on the right side according to the method of Bennett and Xie [32]. Briefly, the right sciatic nerve was exposed at the mid-thigh level and separated from the adjacent tissue. Four loose ligatures using 4–0 chromic gut sutures were made around the dissected nerve with about 1.0 mm interval between each pair of ligatures. The skin wound was closed with sterile silk sutures. The surgical procedure was performed under pentobarbital anesthesia (40 mg/kg, i.p., supplemented as necessary). The formalin injected rats received subcutaneous injections of 100 μL 5% formalin (diluted in 0.9% saline) into the plantar surface of the right hind paw. CFA injected rats were injected subcutaneously into the right hind paw with 100 μL CFA (Sigma-Aldrich) mixed 1 : 1 with normal saline. Age-matched naïve rats with no treatment were used as the control group.

2.2. Immunohistochemistry. Animals (4 rats for each group) were anesthetized on day 7 following the treatments as above with an overdose of pentobarbital sodium, and the rats were euthanized by transcardiac perfusion (250 mL body temperature 0.1 M PBS pH 7.4 followed by 300 mL ice-cold 4% paraformaldehyde/4% sucrose in 0.1 M PB pH 7.4). After perfusion, the lumbar spinal cords (L4–5) were removed, postfixed in 4% paraformaldehyde fixative for 4–6 hours, and placed in a 30% sucrose solution (in 0.1 M PB) over two nights at 4°C. Thirty-micron-thick tissue sections were cut transversely on a cryostat for free-floating immunohistochemical staining for OX-42 (monoclonal mouse anti-rat CD11b, 1 : 200, Serotec, Oxford, UK), Iba-1 (rabbit polyclonal anti-Iba-1, 1 : 400, Abcam, Cambridge, MA), MHC class I antigen (monoclonal mouse anti-rat MHC class I, 1 : 400, Serotec), CD45 (monoclonal mouse anti-rat CD45, 1 : 200, Serotec), and CD68 (monoclonal mouse anti-rat CD68, 1 : 200, Serotec). All of the sections were blocked with 5% normal goat serum in 0.3% Triton X-100 for 1 h at room temperature (RT) and incubated for 48 h at 4°C with the primary antibody. Next, the sections were incubated for 90 min at RT with a corresponding FITC-conjugated secondary antibody. For double labeling, tissues from CCI rats were incubated with a mixture of primary antibody CD45 or CD68 or MHC class I with rabbit anti-Iba-1 (microglia marker, 1 : 2000, Wako), rabbit anti-GFAP (astrocyte marker, 1 : 1000, Chemicon), and rabbit antineutron-specific enolase (NSE, neuronal marker, 1 : 1000, Chemicon). The single-, or double-, stained images were captured with a CCD spot camera and processed using Adobe Photoshop. To analyze MHC class I immunoreactivity, the immunofluorescence intensity was measured as described in our previous reports [9, 31]. The medial portion of the spinal cord dorsal horn was outlined as an area of interest. The average percentage of area covered by MHC class I-immunoreactive (IR) profiles relative to the total outlined area of interest was calculated from three selected tissue sections for each animal. The immunoreactivity level was expressed as the fold increase compared to controls. The rats' sections from all groups were always processed together for comparison under the same conditions.

2.3. Western Blot. Rats (4 rats for each group) were deeply anesthetized and decapitated. The spinal cord segments (L4-5) ipsilateral to the treatment were removed rapidly and homogenized in a RIPA lysis buffer (cell signaling technology; supplemented with 1 mM PMSF, phosphatase, and protease inhibitor cocktail, Sigma). The homogenate was centrifuged at 15,000 g for 40 min at 4°C. The protein concentration of tissue lysates was determined with a BCA Protein Assay Kit (Pierce, Rockford, IL). Fifty microgram aliquots were subjected to SDS-PAGE, and the proteins were electrophoretically transferred to PVDF filters (Millipore, Bedford, MA). After being blocked with 5% nonfat milk in Tris-buffered saline (TBS) containing 0.1% Tween-20 for 1 h at room temperature, the membranes were incubated with an anti-Iba-1 (1 : 1000, in 5% BSA, Wako, Osaka, Japan), anti-CD45 (1 : 200, in 5% BSA, BD Bioscience, San Jose, CA), CD68 (1 : 200, Serotec), and α-tubulin (1 : 1000, Abcam) antibody overnight at 4°C. After washing, the antibody-protein complexes were probed with HRP-conjugated secondary antibody (1 : 10000, Jackson ImmunoResearch, West Grove, PA), developed in ECL solution for 3 min, and exposed onto Kodak hyperfilms. The density of the immunoreactive bands was quantified using NIH ImageJ 1.38 software (NIH, Bethesda, MD), normalized to the density of internal control (e.g., Iba-1/α-tubulin), and expressed as the fold change relative to control group.

2.4. Statistical Analysis. MHC class I immunoreactivity and Iba-1, CD45, and CD68 protein levels were statistically analyzed. All data were reported as the mean ± SEM. Differences

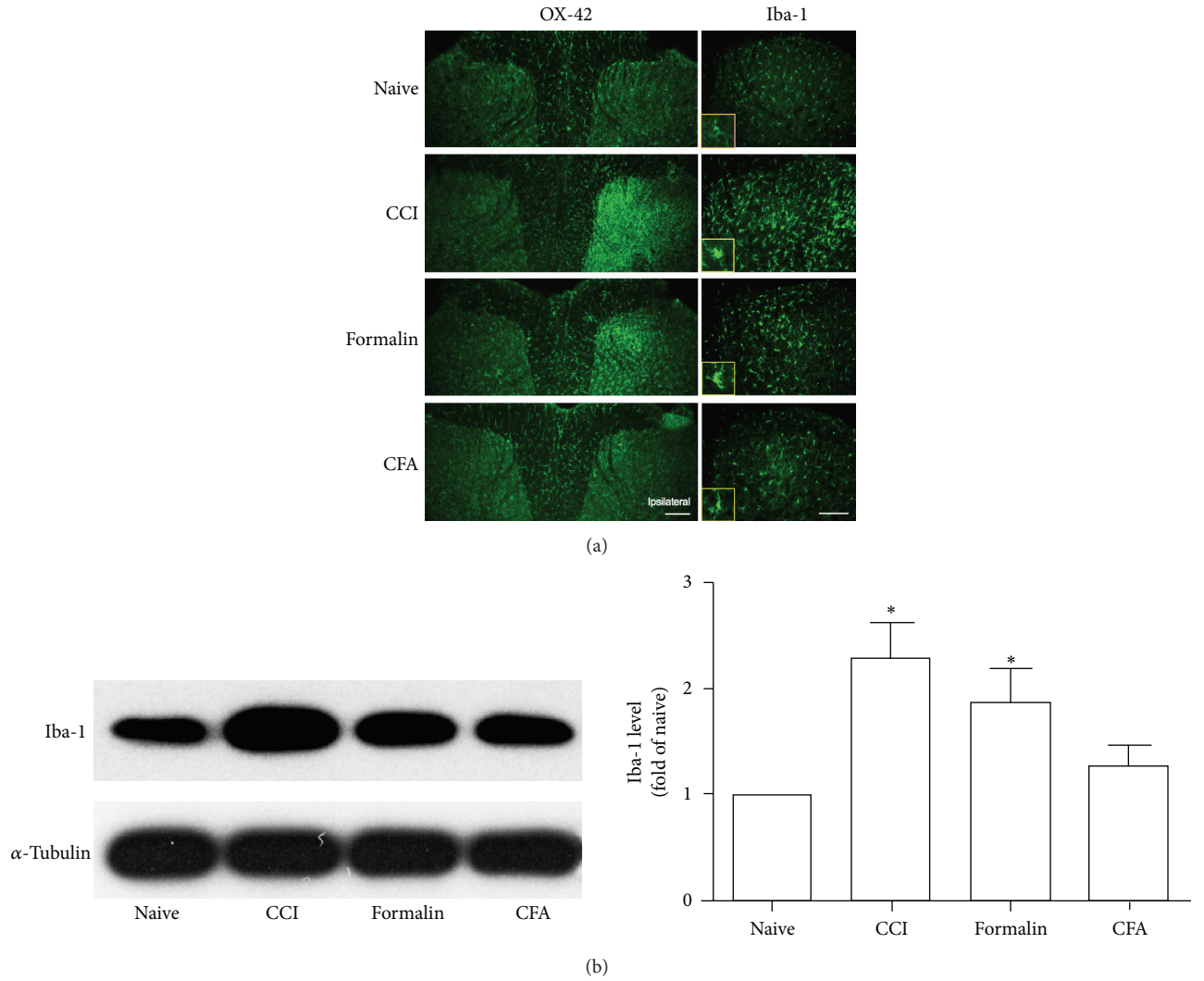

Figure 1: OX-42 and Iba-1 immunoreactivity in the spinal cord dorsal horn after different treatment to rats. A significant increase of OX-42 and Iba-1 expression was present following CCI injury and formalin injection, which was accompanied with obvious morphological change compared with naïve group ((a); Scale bar, 200 μm). The magnified images in yellow box showed the morphology of a single-activated microglia. Representative bands and quantification of western blot analysis (b) showed increased Iba-1 protein level in the lumbar spinal cord after CCI injury and formalin injection, not after CFA injection. $^{*}P < 0.05$, one-way ANOVA and Tukey post hoc test, compared with the naïve (no treatment) control, $n = 4$.

between groups were compared by ANOVA followed by Tukey post hoc analysis (multiple groups). The criterion for statistical significance was $P < 0.05$.

3. Results

3.1. Morphological Changes of Microglial Activation, as well as Increased Expression of the Iba-1 Protein Level, Occur on Day 7 after CCI Injury and Formalin Injection, not Evident in CFA Animals. In naïve rats, microglia labeled by OX-42 and Iba-1 were homogeneously distributed throughout the spinal gray and white matter with a ramified morphology, appearing to be in a resting state (Figure 1(a)). CCI nerve injury and peripheral formalin injection, but not CFA treatment, induced a significant increase of OX-42 and Iba-1 immunoreactivity in the ipsilateral side with morphological changes being a state of clearly hypertrophic (Figure 1(a)). The immunofluorescence result was confirmed by increased Iba-1 protein level demonstrated by Western blot (Figure 1(b)).

3.2. CCI Nerve Injury, Not CFA Injection, Induced Spinal Microglia Upregulation of Immune Molecules, Including CD45, CD68 and MHC I Antigens. Our previous study demonstrated that peripheral formalin injection induced phenotypic changes of microglia with distinct upregulation of CD45 and MHC class I antigens [28]. In the present study, CD45 and MHC class I immunoreactivity were also significantly upregulated on day 7 in the ipsilateral side following CCI injury and formalin injection (Figures 2 and 3). There was a very low basal expression of these two molecules in the CFA model (Figures 2 and 3). We also found that CCI injury induced

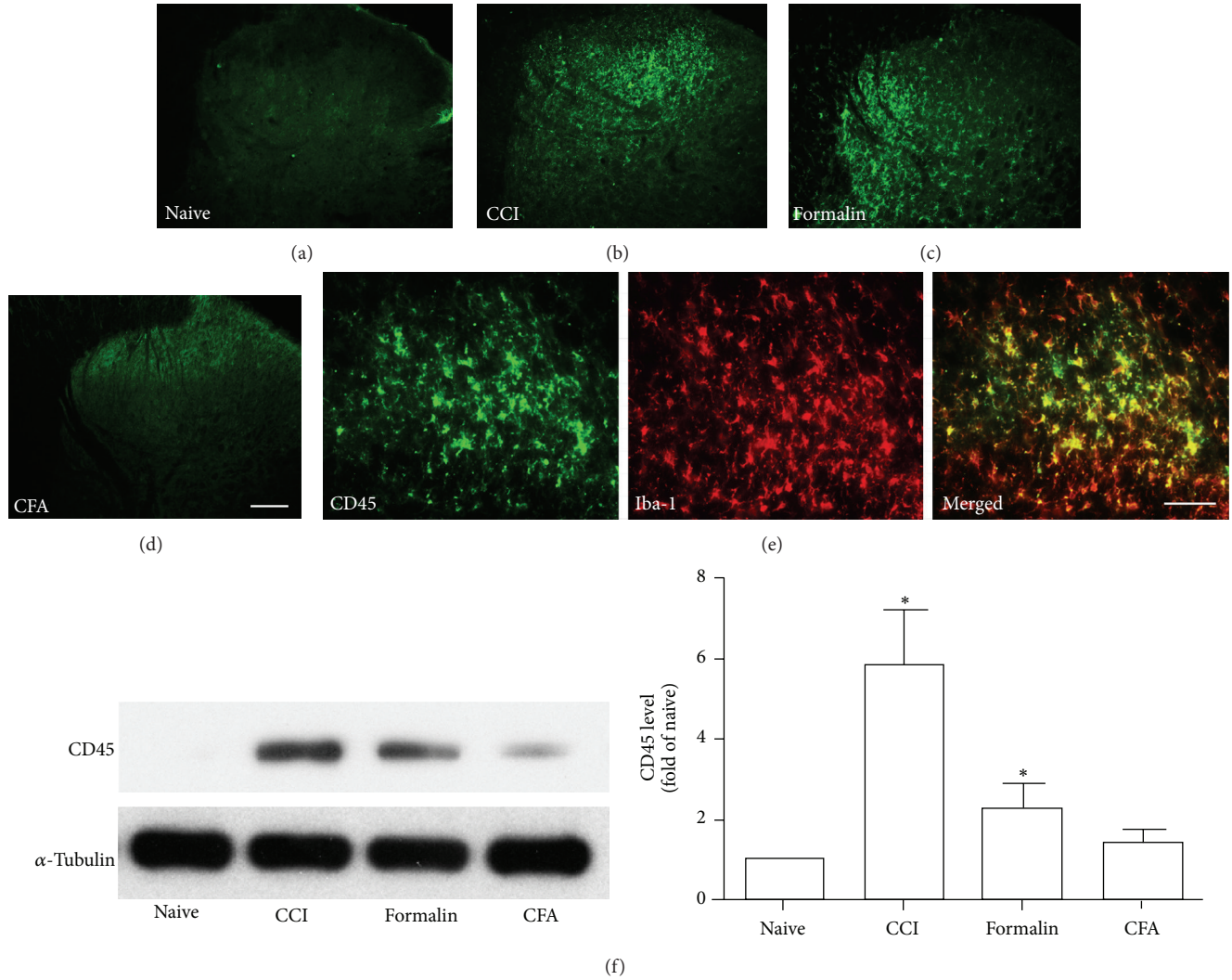

Figure 2: Significant increased expression of CD45 was found in the ipsilateral side of spinal cord of CCI and formalin-injected rats and weak expressed in CFA-injected rats ((a)–(d); Scale bar, 100 μm). Double labeling with Iba-1 confirmed that the cell expressing CD45 is microglia ((e); Scale bar, 50 μm). Representative bands and quantification of western blot analysis (f) showed significantly increased CD45 protein level in the lumbar spinal cord after CCI injury and formalin injection, not after CFA injection.

significant expression of CD68 by immunofluorescence, but not seen in the formalin and CFA groups (Figure 4). The above observation was further supported by western blot analysis (Figures 2(f) and 4(f)). Because a well-proven anti-rat MHC class I antibody for western blot was not available, the immunofluorescence intensity was measured with quantification analysis instead of western blot (Figure 3(f)).

To identify the cell types, we performed double immunostaining with several cell-specific markers: NSE (neurons), GFAP (astrocytes), and Iba-1 (microglia). CD45, CD68, and MHC class I did not colocalize with either NSE or GFAP but mostly colocalized with Iba-1 (Figures 2(e), 3(e), and 4(e)).

4. Discussion

In our previous study, we investigated the possible induction of several microglial surface immune molecules in the spinal cord and found that CD45 and MHC class I antigens were significantly upregulated following peripheral formalin injection into the rat hind paw [28]. The time course of the increased level of CD45 and MHC class I immunoreactivity demonstrated that both CD45 and MHC class I upregulations were evident on day 3 with the peak expression on day 7. In the present study, we compared CD45, CD68 and MHC class I expression on day 7 in three animal pain models using immunohistochemistry and quantitative western blot analysis. Our results clearly indicated that different peripheral tissue injury produced differential phenotypic changes associated with spinal microglial activation. Peripheral nerve injury, not inflammation, induced the activated microglia to acquire these immunomolecular phenotypic changes.

Spinal microglial activation has been extensively reported in almost all animal pain models using OX-42 immunohistochemical marker and mitogen-activated protein kinases

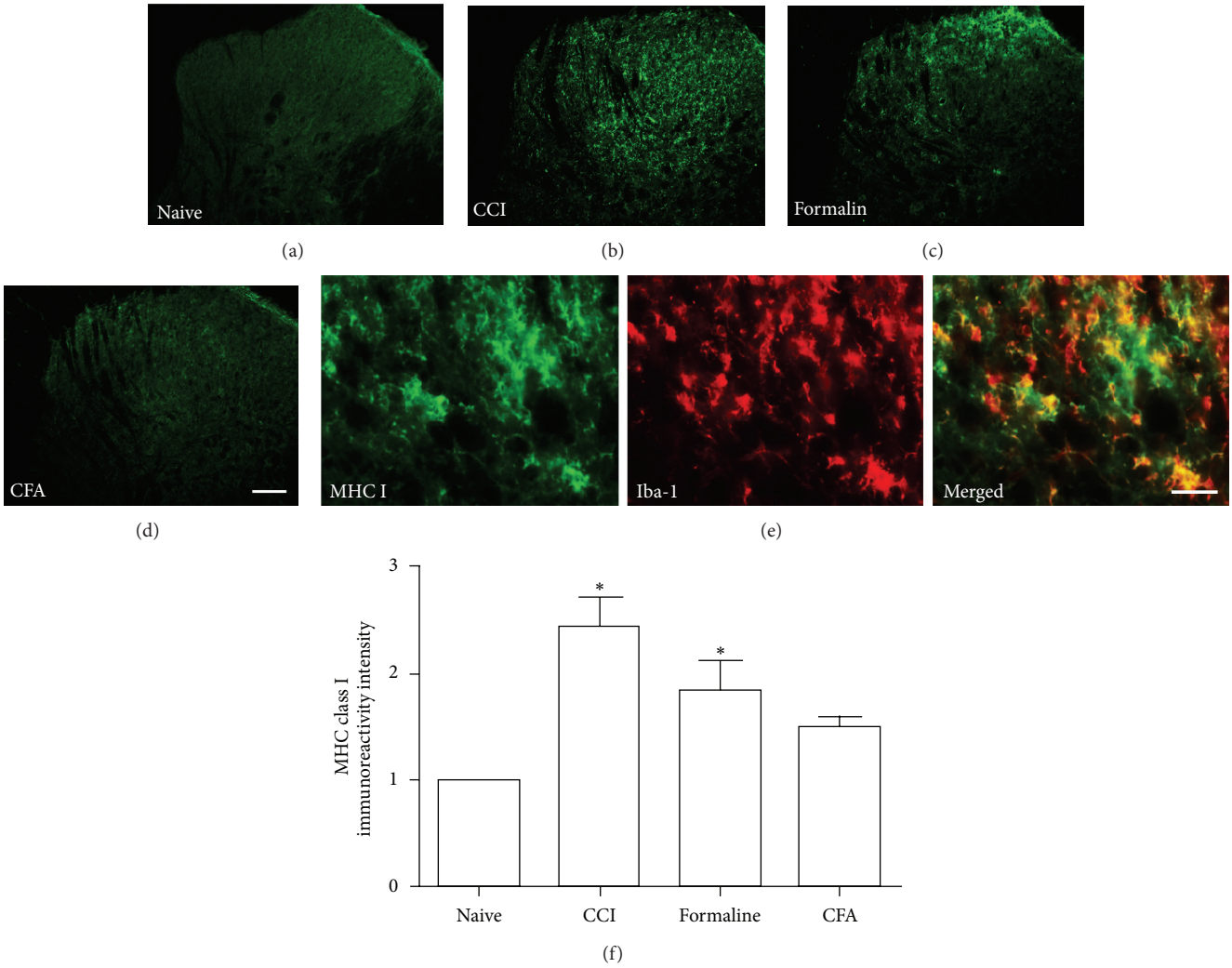

Figure 3: Significant upregulation of MHC class I expression was found in the spinal cord of CCI and formalin-injected rats but weak expressed in CFA-injected rats ((a)–(d) Scale bar, 100 μm). MHC class I positive cells were colocalized with Iba-1 ((e) Scale bar, 30 μm). The intensity of MHC class I immunoreactivity was quantitatively compared among four groups (f). $^{*}P < 0.05$, as compared with the naïve group.

(MAPKs) such as phosphorylated p38 (p-p38) [4–6]. Significant microglial activation with morphological changes was evident as early as 2-3 days, maximal at 7 days, after peripheral tissue injury [7–13, 33]. However, inflammatory pain models produced by intraplantar injection of CFA [31, 34] and muscle pain by intramuscular acidic saline injection [35] caused only minor or no microglial activation. Microglia could be rapidly activated without morphological changes following peripheral inflammation as indicated by the increased expression of p38 MAP kinase in the spinal microglia [36]. It was reported that microglia might undergo at least two distinct stages of activation on the basis of their morphological and phenotypic changes [28]. (1) Early-activated microglia display a "resting" ramified morphology with a relatively small cell body and weakly express molecules normally present in other haematopoietic lineages, such as CD45, MHC class I antigen, and other immunomolecules. (2) Late-activated microglia show upregulation of CD45 and MHC class I and a morphology characterized by the hypertrophic cell body and the shortening of cellular processes. In the present study in CCI model, activated microglia showed the strongest upregulation of immune molecules, such as CD45, CD68, and MHC class I with a robust degree of morphological change being observed, but CFA-induced inflammation did not. Peripheral formalin injection also produced significant microglial activation and upregulation of some immune molecules. As we discussed in our previous publication [37], the formalin test is both a short-term inflammatory and a long-term nerve injury pain model. A 5% formalin injection damages the local nociceptive receptors at the injection site and results in the loss of responses to thermal and mechanical stimuli [31]. Peripheral formalin injection damaged nerve endings and produced spinal microglial activation. However, the damage was mild if compared to CCI injury, the spinal microglial activation was not stronger than CCI and did not induce significant upregulation of CD68. These findings suggested

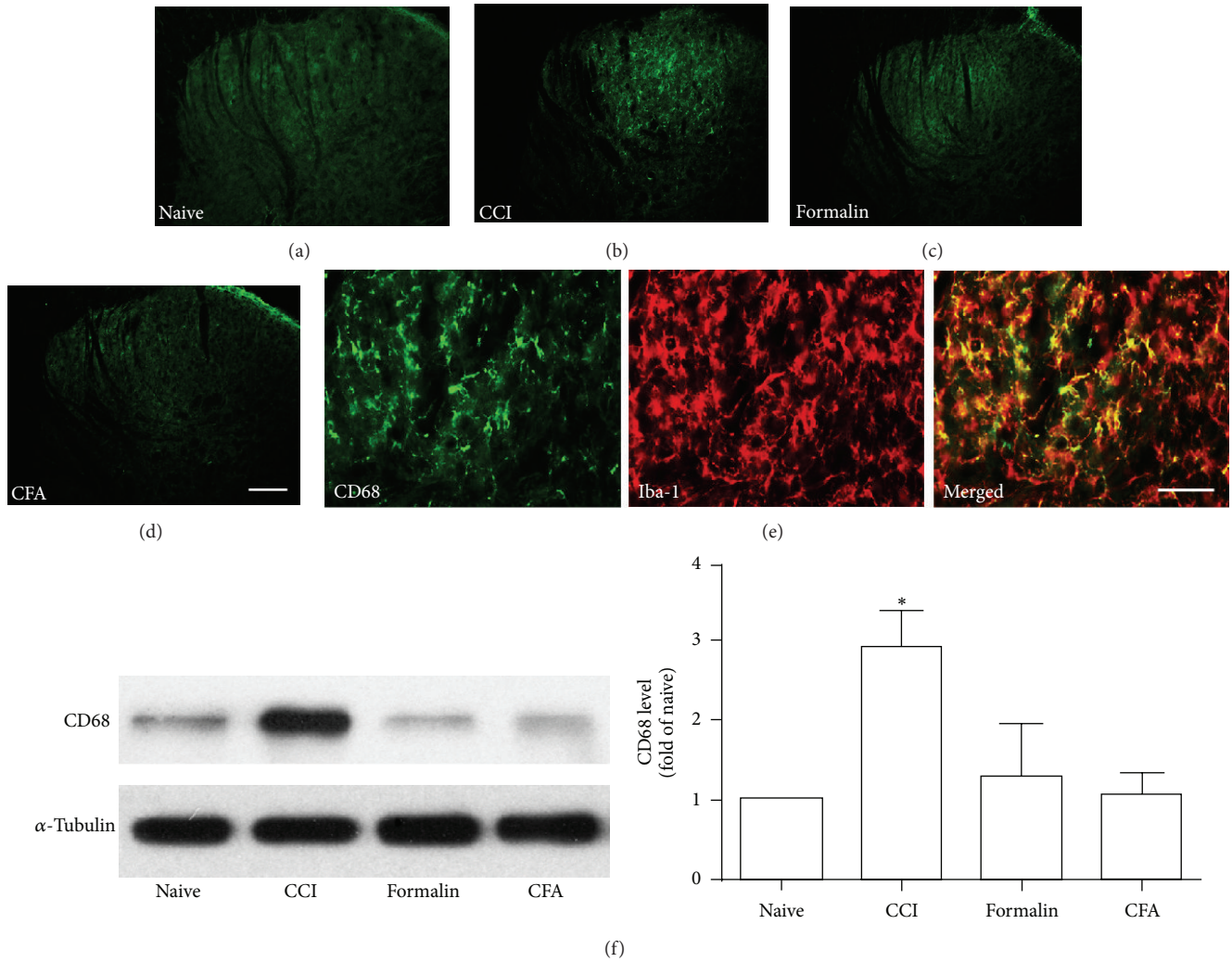

Figure 4: A significant increase of CD68 expression was present only following CCI injury, which was weak after formalin and CFA injection ((a)–(d); Scale bar, 100 μm). CD68 positive cells were colocalized with Iba-1 ((e); Scale bar, 50 μm). Representative bands of Western blot and quantification of Western blot analysis (f) showed increased level of CD68 in CCI group. $^{*}P < 0.05$ as compared with the naïve group, $n = 4$.

that spinal microglial activation and cell surface immune molecular expression were dependent on the pathogenic stimulus administered; peripheral nerve injury caused the late-activated microglia to acquire immune phenotypic and morphological changes. However, molecular mechanisms for the spinal microglial activation by peripheral nerve injury are still unknown. Inflammation/immune response at the site of the injured sciatic nerve has been well elucidated, but there is no report on the inflammatory status of the ipsilateral side of spinal nerve following peripheral injection of CFA or formalin into the animal's hind paw. Whether inflammation/immune response of the ipsilateral side of spinal nerve is an essential trigger for the spinal microglial activation is worth further doing.

5. Conclusions

With different pain conditions, microglia could exist in differential statuses of activation. The immunomolecular and phenotypic changes of microglia are associated with nerve injury in most cases. For this reason, elucidation of the different activation modalities of microglia could push us to find more specific and promising targets for inhibiting microglial activation associated pathological pain.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgments

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Review Article

Involvement of MicroRNA in Microglia-Mediated Immune Response

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MicroRNAs (miRNAs) are an abundant class of small noncoding RNA molecules that play an important role in the regulation of gene expression at the posttranscriptional level. Due to their ability to simultaneously modulate the fate of different genes, these molecules are particularly well suited to act as key regulators during immune cell differentiation and activation, and their dysfunction can contribute to pathological conditions associated with neuroinflammation. Recent studies have addressed the role of miRNAs in the differentiation of progenitor cells into microglia and in the activation process, aiming at clarifying the origin of adult microglia cells and the contribution of the central nervous system (CNS) environment to microglia phenotype, in health and disease. Altered expression of several miRNAs has been associated with Alzheimer's disease, multiple sclerosis, and ischemic injury, hence strongly advocating the use of these small molecules as disease markers and new therapeutic targets. This review summarizes the recent advances in the field of miRNA-mediated regulation of microglia development and activation. We discuss the role of specific miRNAs in the maintenance and switching of microglia activation states and illustrate the potential of this class of nucleic acids both as biomarkers of inflammation and new therapeutic tools for the modulation of microglia behavior in the CNS.

1. Introduction

Microglia cells are crucial for the development and maintenance of the central nervous system (CNS). In addition to acting as sensors of environmental changes that precede pathological events, these cells have been shown to support neuronal function by monitoring synaptic activity, controlling synaptogenesis, and promoting neuronal apoptosis during development [1–3]. Although they are considered one of the four major cellular types of the CNS, they do not originate from the same precursor cells as astrocytes, oligodendrocytes, or neurons. Instead, they derive from myeloid progenitor cells and share several markers with peripheral monocytes, macrophages, and dendritic cells, such as CD11b, F4/80, and CD45 [4]. The first resident parenchymal microglia cells are believed to originate from yolk sac immature macrophages in early stages of fetal development. In humans, microglia precursor cells arrive at the brain in two waves during the first and second trimester of gestation, while in rodents this migration occurs shortly before and after birth.

The sudden increase in $CD11b^+$ and $F4/80^+$ cells observed in the early postnatal period in rodents was until recently attributed to the recruitment of bone marrow derived cells, suggesting that myeloid precursors could also contribute to the initial pool of microglia cells in the CNS. However, most of the studies supporting these findings used irradiation of the recipient animals to allow bone marrow engraftment of genetically-labeled cells [5], which was later found to strongly influence the observed results [6]. In 2010, Ginhoux and colleagues shed light on the origin of microglia. The authors performed *in vivo* lineage tracing studies using Cre recombinase activity, which was induced into pregnant mice between days 7 and 8 of fetal development, when embryonic hematopoiesis is restricted to the yolk sac. The results from

this study clearly demonstrated that postnatal hematopoietic progenitors do not contribute significantly to microglia postnatal numbers and that the cellular expansion observed in this period is mainly dependent on the proliferation of the resident yolk sac-derived microglia population [7].

The question remains whether this is also true in the adult brain, especially following a neurological insult or in the case of a neurodegenerative disease, wherein the integrity of the blood-brain barrier (BBB) may be compromised. Several studies have shown that the infiltration of bone marrow-derived cells into the brain is possible under those circumstances and may even play a central role in disease modulation. Nevertheless, the exact nature of the contribution of parenchymal and blood-derived microglia to the neuroimmune response, in the context of neuronal disease, remains to be clarified [8–10].

Following their migration to the neuronal tissue, microglia cells assume a surveying phenotype, usually referred as "resting microglia," characterized by a small and static cell body, a large number of highly motile ramifications, and low expression of macrophage-related surface markers, such as the major histocompatibility complex II (MHC II) and CD45 [11]. The low levels of these markers distinguish parenchymal "resting" microglia from peripheral macrophages. However, following a neuronal insult, such as ischemia, infection, and trauma or in the presence of inflammatory mediators (IFN-γ), microglia cells assume an amoeboid form, losing their ramifications, and overexpressing the above-mentioned markers. This process is referred to as microglia "activation" and is known to induce profound phenotypical changes, making parenchymal microglia to become almost indistinguishable from peripheral macrophages [11].

Similarly to what has been described in macrophages, microglia activation can also originate different subsets of cells, depending on the nature of the activating stimulus and surrounding environment. These different activation phenotypes express distinct molecular markers and exert different functions in the neuronal tissue [12]. The definition of the different activation states of macrophages was initially based on the expression of proinflammatory receptors and cytokines (M1 phenotype—classical activation) or on the expression of anti-inflammatory receptors and cytokines (M2 phenotype—alternative activation). Further studies revealed the existence of several intermediate activation states, which led to the conclusion that these two basic phenotypes can overlap and that macrophages are able to assume a broad spectrum of phenotypes that cannot be oversimplified and separated into discrete categories.

Contrarily to macrophages, the mechanisms responsible for microglia phenotype regulation in the CNS are poorly understood. M1 activation, which is characterized by an increase in the production of IL-1β, IL-6, TNF-α, and nitric oxide (NO), has been identified following acute brain injury caused by trauma or stroke [13, 14]. However, in the context of neurodegenerative disorders, the distinction between the M1 and M2 phenotypes has proven to be more challenging. While several studies have identified the presence of M2 markers, such as TGF-β and IL-10, in the brain of Alzheimer's disease (AD) animal models, as well as an increase in the expression of M2 genes AG1 (arginase 1) and CHI3L1/CHI3L2 (chitinase 3-like 1/2) in AD patients [15], inflammation in the human AD brain has also been associated with upregulation of IL-6, IL-1β, and TNF-α, all markers of the M1 state, in the vicinity of amyloid deposits [16, 17]. Most authors believe that the M2-like phenotypes are less aggressive to the neuronal tissue, promoting tissue repair and phagocytosis of protein aggregates and cell debris, while the M1-like phenotypes are more prone to induce neuronal toxicity by themselves, due to the high expression of inflammatory mediators and NO. Whether human microglia can switch from an M2 to an M1 phenotype with a detrimental effect to the brain is still not clear. However, several studies have pointed to the possibility of microglia "priming," a phenomenon associated with age and chronic inflammation, in which exposure to low levels of systemic signaling molecules can exacerbate microglia response to a second local stimulus, such as the presence of Aβ aggregates, potentiating the development of tissue damaging phenotypes [16]. Although some of the molecular intervenients and exogenous stressors underlying microglia activation *in vitro* have been identified [18–20], in more complex environments, such as the diseased brain, there is still a lack of answers concerning the molecular mechanisms responsible for microglia phenotypic changes [21]. This led several scientists to propose a role for certain key transcription factors and microRNAs (miRNAs) in these processes [22].

2. MiRNA Biogenesis and Activity

MiRNAs are transcribed from intragenic or intergenic regions by RNA polymerase II or RNA polymerase III, originating large stem-loop hairpin structures, designated pri-miRNAs [23]. These structures, which are asymmetrically cleaved by an enzymatic complex containing Drosha, a RNAse III endonuclease, originate hairpin-structured precursors designated pre-miRNA [24, 25]. Alternatively, non-canonical pathways for pre-miRNA biogenesis can occur, such as the production of mirtrons, which consist in pre-miRNAs generated through the direct splicing of introns [26].

Pre-miRNAs are exported to the cytoplasm by the complex Exportin-5/RanGTP, which recognizes and binds the characteristic overhangs of pre-miRNAs. These precursors are then incorporated into a processing complex containing another RNAse III endonuclease (Dicer), which is responsible for removing the stem-loop of the precursor, originating a double-stranded miRNA molecule with 3′ overhangs (mature miRNA). This duplex molecule is then incorporated into the RNA-induced silencing complex (RISC) or the microribonucleoprotein complex (miRNP), where the strand with the least thermodynamically stable 5′ end is used to activate the complex and guide it to complementary mRNA targets [27]. During miRNA-mediated posttranscriptional gene regulation, the RISC complex, containing the single stranded miRNA template, binds mainly to the 3′ untranslated region (3′ UTR) of the target mRNA, in one or more locations. The complementarity between the mRNA and the nucleotides 2–8 on the 5′ region of the miRNA (seed region) is responsible for this binding and allows the recognition of multiple mRNA

targets by a single miRNA molecule [24]. MiRNA-mediated regulation of gene expression is achieved either by translation repression or degradation of the mRNA target molecule [28] and has been associated with many important biological processes, including inflammation, apoptosis, angiogenesis, development, proliferation, patterning, and differentiation.

3. MiRNA Role in Microglia Development

The mechanism of miRNA-mediated posttranscriptional regulation is very well suited to the control of fate-changing cellular events, such as differentiation and activation. Since these processes usually involve changes in several proteins and different signaling pathways, such control can be easily achieved through the expression of a single miRNA or a set of specific miRNAs. In addition, miRNAs can directly target transcription factors, the master regulators of most cellular events. A number of studies have revealed that miRNAs may provide a genetic switch to inactivate a set of target genes through the regulation of a specific transcription factor, while miRNA expression can be regulated by transcription factors that bind upstream of pre-miRNA genes [22, 29]. Therefore, these two classes of regulators can work together to orchestrate complex cellular events, such as monocyte-macrophage differentiation and microglia development from primitive macrophages in the yolk sac or bone marrow precursors.

Two transcription factors, namely, CEBPα and PU.1, have been shown to be critical for monocyte/macrophage differentiation and microglia development, CEBPα being considered the master regulator of hematopoietic stem cell differentiation. Several miRNAs are controlled directly by CEBPα, including miR-223, which is also regulated by the transcription factor NFI-A. However, while CEBPα promotes miR-223 expression, NFI-A inhibits the expression of this miRNA [30], which represses NFI-A through a feedback loop. Therefore, when CEBPα levels are high, miR-223 expression is enhanced, and NFI-A levels decrease, promoting granulocyte differentiation, but when NFI-A levels are relatively high and miR-223 levels are low, other pathways are favored, such as monocyte differentiation.

On the other hand, PU.1 is required to promote the skewing of granulocyte-macrophage progenitors to the monocyte lineage. PU.1 levels, although directly controlled by CEBPα, have to be relatively high when compared to CEBPα levels, to avoid favoring the granulocyte lineage. MiR-424 is upregulated by PU.1 and, through the targeting of NFI-A, upregulates the expression of the M-CSF receptor, skewing differentiation towards the monocyte/macrophage lineage in the bone marrow [31]. Forrest and coworkers have demonstrated that, in addition to miR-424, miR-222, miR-155, and miR-503 play an important role in monocyte differentiation through combinatorial regulation [31]. These authors have shown that when overexpressed, these miRNAs are able to cause cell-cycle arrest and partial differentiation in THP-1 cells (leukemia model). MiR-222 and miR-155 caused G2 arrest and apoptosis, while miR-424 and miR-503 caused G1 arrest and the downregulation of miR-9, an antidifferentiative miRNA, by targeting a site in its primary transcript.

In another recent study, the oncogenic miR-17-92 cluster (which carries six miRNAs: −17, −18a, −19a, −20a, −19b-1, and −92a) has been directly associated with the process of monocyte to macrophage differentiation, in which PU.1 and Egr2 are also involved [32]. Upon differentiation into macrophages, the transcription factor PU.1 was found to induce the secondary determinant Egr2 which, in turn, directly represses miR-17-92 expression by promoting histone H3 demethylation within the CpG island of the miR-17-92 promoter. Conversely, Egr2 itself is targeted by miR-17-92, indicating the existence of a mutual regulatory relationship between miR-17-92 and Egr2 [32]. Given the similarities between macrophages and microglia, it is reasonable to assume that some of these miRNAs and transcription factors also play an important role in microglia differentiation in the brain, and it would be interesting to ascertain whether the same regulatory loops might exist in yolk sac-derived microglia.

So far, the only study addressing miRNA contribution to microglia development was performed by Ponomarev and colleagues [33, 34]. These authors showed that miR-124, one of the most abundant miRNAs in the brain, is required to maintain the quiescent state of microglia in the brain. By targeting the transcription factor CEBPα and the cyclins CDK4 and CDK6, miR-124 is able to reduce the expression of PU.1 and its downstream target, the M-CSF receptor, restricting cellular proliferation and potentiating the differentiation of primitive macrophages to adult microglia in the brain [33]. While during the first two weeks after birth, microglia isolated from the brain presented low levels of miR-124 and a $CD45^{high}$/MHC class II^{high} phenotype, characteristic of active and proliferating cells, adult microglia presented the opposite phenotype, $CD45^{low}$/MHC class II^{low}/miR-124^{high} [33, 34]. The authors hypothesized that the high levels of miR-124 observed in adult microglia are a specific consequence of the CNS environment. This idea was based on their observation that sublethally irradiated mice, transplanted with bone marrow GFP^+ progenitor cells exhibiting a $CD45^{high}$/MHC class II^{high}/miR-124^{low} phenotype, presented GFP^+ $CD11b^+$ positive cells in the brain with a $CD45^{low}$/MHC class II^{low}/miR-124^{high} phenotype. To confirm this hypothesis, Ponomarev and colleagues cocultured bone-marrow-derived macrophages with astroglial or neuronal cell lines, and they observed, in both cases, a downregulation of MHC class II and CD45 levels as well as an upregulation of miR-124 expression. Several suggestions were made concerning the mechanisms underlying miR-124 upregulation in microglia, including the direct transfer of miR-124 from neuronal cells to microglia through exosomal shuttle vesicles, direct cell-to-cell contact between these two cell types and the release of anti-inflammatory factors, such as CX3CL1 and TGF-β, by neuronal cells [34, 35].

4. MiRNA Role in Microglia Activation

In addition to being involved in the regulation of the differentiation process of microglia, several studies suggest a role for miRNAs as modulators of M1 and M2 polarization in both

microglia and macrophages. MiR-155, broadly considered a proinflammatory miRNA, was one of the first miRNAs to be directly linked to the M1 phenotype (Figure 1). This miRNA was shown to be upregulated in macrophages, monocytes, and microglia in response to several proinflammatory stimuli, such as LPS, IFN-γ, and TNF-α [18, 36, 37]. In this regard, we have recently shown that miR-155 targets anti-inflammatory proteins in microglia, such as the suppressor of cytokine signaling 1 (SOCS-1), leading to the upregulation of several inflammatory mediators characteristic of the M1 phenotype, including the inducible nitrogen synthase (iNOS), IL-6, and TNF-α [18], as described in Figure 1. In addition, miR-155 upregulation increases the expression of IFN-β, which is probably related with a feedback mechanism to control the immune response, since IFN-β is known to upregulate the expression of SOCS-1 and IL-10, two important anti-inflammatory mediators [38, 39]. Our results confirm that miR-155 upregulation contributes to a microglia-mediated neurotoxic response, which has been largely associated with the M1 phenotype. Furthermore, recent studies have shown that miR-155 is able to target M2-associated genes, such as that encoding SMAD2, a protein involved in the TGF-β pathway [40], and CEBPβ, a transcription factor important for the expression of IL-10, arginase 1, and CD206 [41], further supporting the hypothesis that miR-155 is required to skew microglia activation to M1-like phenotypes.

Several other miRNAs have been directly related to the M1 phenotype, including miR-101 and miR-125b. Zhu and coworkers observed an upregulation in miR-101 levels in response to several TLR ligands in macrophages. The overexpression of this miRNA resulted in the downregulation of MAPK phosphatase 1 (MPK-1), promoting the activation of MAPK and the expression of M1-associated proinflammatory cytokines, such as IL-6, TNF-α, and IL-1. On the other hand, miR-101 inhibition enhanced MPK-1 expression and decreased p38 and JNK activation [42]. Regarding miR-125b, Chaudhuri and colleagues reported an increase in M1 macrophage activation, with upregulation of MHC class II, CD40, CD80, and CD86, upon overexpression of this miRNA. The authors related these effects to miR-125 targeting of interferon regulatory factor 4 (IRF4) and also observed that, upon forced expression of miR-125b, macrophages adopted an M1 cytotoxic phenotype, presented elevated responsiveness to IFN-γ, and were more effective in killing EL4 T-lymphoma tumor cells *in vitro* and *in vivo* [43].

In contrast to miR-101 and miR-125b, miR-92a was recently shown to be downregulated in response to the activation of different TLRs, this decrease being necessary to enhance TLR-triggered production of inflammatory cytokines in macrophages [44]. On the other hand, miR-92a overexpression inhibited the activation of the JNK/C-JUN pathway through the targeting of mitogen-activated protein kinase kinase 4 (MKK4), which activates JNK/stress-activated protein kinase and a direct target of miR-92a. Therefore, miR-92a downregulation can be considered as an additional requirement for M1 macrophage activation [44].

Finally, as illustrated in Figure 1, miR-124 has been reported to contribute to the M2 phenotype of macrophages and microglia, since its forced overexpression led to the downregulation of M1-associated markers, such as IL-6, TNF-α, and iNOS, and to an increase of proteins associated with the M2 phenotype, as is the case of TGF-β, arginase-1, and FIZZ1 [33]. It was concluded that, in microglia, an inverse correlation exists between the expression of M1 activation markers and that of miR-124 (Figure 1). Indeed, the highest levels of miR-124 were detected in $CD45^{low}$/MHC class II^{low} nonactivated resident microglia, but miR-124 expression decreased dramatically upon cell treatment with IFN-γ and GM-CSF, two stimulus known to upregulate CD45 and MHC class II expression and to potentiate the M1 phenotype [33]. Table 1 lists several miRNAs involved in microglia activation, indicating the phenotype (M1 or M2) favored by their up- or downregulation.

Taken together, the above-mentioned results highlight the prominent role of miRNAs in the regulation of the activation of both microglia and macrophages and open new possibilities in the field of anti-inflammatory therapies. Using appropriate gene therapy tools, miRNA modulation could be an interesting and promising strategy to fine-tune the immune response, skewing cell activation to the M1 or M2 phenotypes according to the specific requirements of each disease setting.

5. MiRNA Dysregulation in Neurodegeneration and Neuroinflammation

Neurodegeneration is characterized by neuronal loss of specific neuronal circuits associated with cognitive and motor functions. In this context, neuronal death is considered both cause and consequence of neuroinflammation, a process involving microglia and astrocyte proliferation and activation. The excessive production of inflammatory mediators by these cells propagates inflammation in the brain and contributes to the triggering of a local and systemic immune response, which is characterized by changes in miRNA levels in the nervous tissue and in the periphery. Although the traffic of peripheral mononuclear cells from the blood stream to the CNS is tightly regulated at the level of the BBB, it is known that the integrity of this barrier is highly compromised in severe brain diseases, such as stroke, brain trauma [9], and AD [5, 45, 46]. The local disruption of the BBB in a disease context facilitates the passage of blood-derived cells to the nervous tissue. Therefore, both microglia and peripheral mononuclear phagocytes are able to impact the pathoetiology of neurodegenerative diseases, and miRNAs presenting altered expression levels in both of these cell types can be used as new potential biomarkers and targets of neurodegeneration.

Through the cellular crosstalk between the brain and the periphery, the nervous system can influence peripheral immune functions, and, conversely, the immune system can affect neuronal activity [47]. A specific subset of miRNAs is able to regulate both cognitive and immune features, but the neuroimmune impact of these molecules is far from being fully understood. However, the role of miRNAs in neuroimmune pathologies has recently started to be unveiled. In the past few years, a large number of studies have emerged

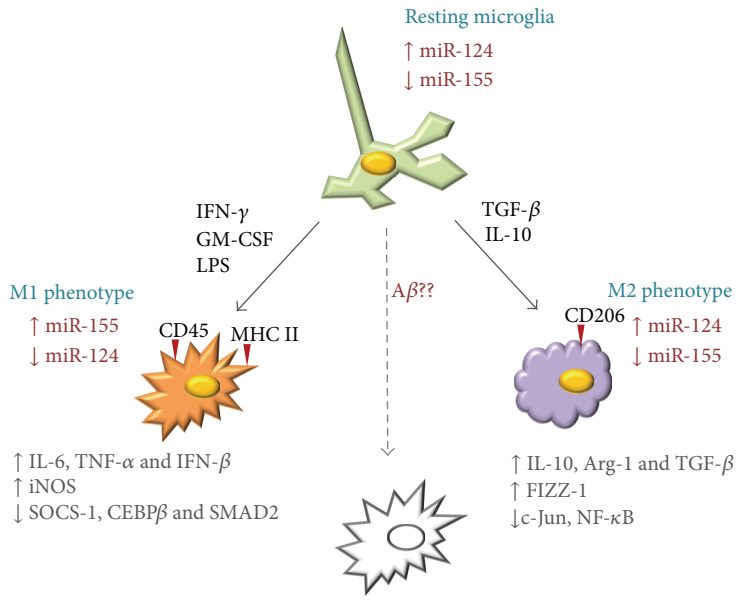

Figure 1: MiRNA contribution to microglia activation phenotypes. Resting microglia cells are characterized by low expression levels of miR-155 and a relatively high expression of miR-124. In the presence of strong inflammatory stimuli, such as IFN-γ, GM-CSF, and LPS, microglia assume a classical activation phenotype (M1), characterized by the upregulation of both CD45 and MHC II, the expression of several inflammatory mediators, such as iNOS, the inflammatory cytokines IL-6 and TNF-α, the type I interferon IFN-β, and the downregulation of miR-124. MiR-155 upregulation is thought to be crucial for the establishment of this phenotype, since this miRNA directly targets several anti-inflammatory molecules, including SOCS-1. Alternatively, in the presence of TGF-β or the anti-inflammatory cytokine IL-10, a different activation phenotype is observed (M2). In this case, CD206 is upregulated at the cell surface, and anti-inflammatory molecules involved in tissue repair and angiogenesis are expressed. Moreover, most proinflammatory pathways, including those regulated by the transcription factors c-Jun and NF-κB, are inactivated, and miR-155 upregulation is not observed. Certain endogenous danger signals associated with neurodegenerative disorders, such as Aβ fibrils present in senile plaques of AD patients, can also cause microglia activation, although the exact nature of the observed phenotypic changes is yet to be fully characterized.

Table 1: MiRNAs in microglia and macrophage activation.

	Role	Phenotype	Ref
↑ miR-155	Targets SOCS-1 causing ↑ of iNOS, TNF-α, IL-6, and IFN-β Targets SMAD2 and CEBPβ	M1	[18, 36, 40]
↑ miR-124	Lead to ↓ IL-6, iNOS, and TNF-α and ↑ TGF-β, arginase-1, and FIZZ1	M2	[33, 34]
↑ miR-101	↓ MPK-1 promoting MAPK activation and IL-6, TNF-α, and IL-1 expression	M1	[42]
↑ miR-125b	↑ MHC class II, CD40, CD80, and CD86 through targeting of IFR4 Elevates responsiveness to IFN-γ	M1	[43]
↓ miR-92a	Inhibited activation of JNK/c-Jun through targeting of MKK4 Its downregulation is necessary to promote the M1 phenotype	M1	[44]

reporting the disruption of miRNA expression during neuroinflammation and neurodegeneration processes [48]. For example, miR-146, which has been directly related with AD [49] and epilepsy [50], has also been shown to be highly overexpressed in Aβ and TNF-α stressed human microglia cells, and this effect was inversely correlated with the levels of inflammation-related proteins, such as CFH and IRAK-1 [51]. Another brain enriched miRNA, miR-124, which has been directly related with the maintenance of the quiescent state of microglia [33], has also been shown to inhibit the neuronal transcription regulator complex REST, which is involved in Rett syndrome [52] and neuronal changes during chronic cocaine intake [53].

MiRNA dysregulation in the context of neurodegeneration can be a consequence of genetic or sporadic anomalies. In addition, the miRNA-related machinery can be impaired

in neurological disorders [54]. Accordingly, altered miRNA profiles have been identified in several neurodegenerative diseases. Striatal miR-22, miR-29c, miR-128, miR-132, miR-138, miR-218, miR-222, miR-344, and miR-674-3p, as well as the cellular levels of Drosha were shown to be downregulated in both YAC128 and R6/2 transgenic mouse models of Huntington's disease (HD) [55]. In the frontal cortex and striatum of human HD brains, several miRNAs were found to be altered with respect to the brains of healthy subjects, and changes in the primary nucleotide structure of the 3′ terminus of certain miRNAs were also reported [56]. MiRNA profiling studies in Parkinson's disease (PD) revealed an early downregulation of miR-34b/c in human brain areas, with variable neuropathological effects at clinical (motor) stages [57], while in ALS mouse models deficiency in miR-206 accelerated disease progression [58].

A large number of studies using cellular and mouse models, human hippocampus, human cortex, and whole brain samples have revealed altered miRNA expression profiles in AD. MiR-106b was found to be aberrantly expressed in the APPswe/PSΔE9 AD mouse model, and its levels were correlated with the dysregulated expression of the TGF-β type II receptor [59]. Wang and coworkers have shown that the exposure of SH-SY5Y cells to Aβ_{1-42} oligomers leads to the increase of miR-106b expression and to the consequent impairment in TGF-β signaling, providing a possible explanation for the observed neurodegeneration [59]. As mentioned before, TGF-β signaling is a marker of the M2 microglia phenotype, and, as such, miR-106b overexpression could be considered an additional cause of microglia skewing to the M1 phenotype following Aβ exposure.

Interestingly, Schonrock and collaborators have shown that half of the tested miRNAs in their study were downregulated in hippocampal neuronal cultures in response to Aβ_{1-42} peptides and that the dysregulated miRNAs were likely to affect target genes belonging to signaling pathways known to be disrupted in AD. These results were further validated in the hippocampus of APP23 mice and human AD brains [60]. Curiously, many of those miRNAs, as miR-21, miR-146a, let-7i, and miR-125b, had already been associated with inflammation.

Although the majority of miRNA profiling studies in neurodegenerative diseases has been performed in brain samples, miRNA dysregulation has been found in other biological sources, such as plasma, peripheral blood, and cerebral spinal fluid (CSF). For example, miR-34a was shown to be significantly increased in plasma from HD gene carriers prior to symptom onset, suggesting that plasma miRNAs can be used as biomarkers in HD [61]. In another study, eighteen miRNAs were found to be differentially expressed in peripheral blood mononuclear cells of nineteen PD patients with respect to miRNA levels in thirteen control subjects [62]. In blood serum from AD patients, miR-137, miR-181c, miR-9, and miR-29a/b were found to be downregulated, and although the ability of these miRNAs to conclusively diagnose AD is currently unknown, these blood-circulating miRNAs have potential to be used as additional biomarkers of the disease [63]. Although more difficult to obtain, CSF has also been considered a source of biomarkers for many neurological diseases, since this fluid constantly exchanges material with the brain parenchyma and is less prone to the influence of peripheral organs. Alexandrov and collaborators analyzed miRNA abundance in the CSF of AD patients and observed a significant increase in the levels of miR-9, miR-125b, miR-146a, and miR-155, with respect to age-matched controls. Interestingly, these miRNAs are NF-κB-sensitive proinflammatory miRNAs, also known to be upregulated in AD brains and have been associated with progressive spreading of neuroinflammation [64]. Taken together, these results indicate that the effective application of miRNAs as biomarkers for neurodegenerative diseases should include miRNA profiling, not only in the blood but also in serum, plasma, and different cellular subtypes, as well as the parallel correlation of the obtained results with brain morphology overtime, in order to overcome clinical issues related with disease staging and progression.

MiRNA deregulation has also been associated with viral-induced neuroinflammation and neurodegenerative processes. Mishra and coworkers showed that human microglia cells treated with HIV-1 Tat-C protein, a molecule involved in neuroinflammation in HIV-infected patients, present increased miR-32 expression with consequent changes in the levels of the downstream target TRAF3, which, in turn, was found to control IRF3 and IRF7 expression [65]. It was also demonstrated that miR-146a is upregulated in human microglia cells under HIV infection, regulating the inflammatory response by targeting the CCL8 [66] chemokine. Therefore, it seems that miRNA expression is altered after inflammation in immune and glial cells in order to support the fine-tuning of immune functions essential to maintain brain homeostasis. An interesting study by Dave and Khalili showed that morphine-induced inflammation and oxidative stress in human monocyte-derived macrophages contribute to the expansion of the HIV-1 viral reservoir in the CNS and HIV-associated dementia [67]. The authors provided evidence that this process is regulated by differentially expressed miRNAs, including miR-15b and miR-181b, both linked to several targets in the proinflammatory pathways.

MiRNAs are also believed to modulate microglia inflammation after hypoxia/ischemia, which may contribute to neuronal damage. Hypoxia causes upregulation of the Fas ligand (FasL) and simultaneously downregulation of miR-21 in microglia, influencing neuronal apoptosis. Interestingly, according to the work of Zhang and colleagues, the ectopic expression of miR-21 partially protects neurons from cell death caused by hypoxia-activated microglia [68]. The same authors reported a potential role for miR-181c in the regulation of TNF-α expression after hypoxia/ischemia and microglia-mediated neuronal injury. They showed that oxygen-glucose deprivation (OGD) induces microglia activation *in vitro* and in the hippocampus of Wistar rats (four-vessel occlusion—4-VO—rat model of ischemia), as concluded by observation of the overproduction of TNF-α, IL-1β, and NO and the downregulation of miR-181c. On the other hand, the heterologous expression of this miRNA was found to protect neurons from cell death caused by OGD-activated microglia [69].

Regarding neurodegenerative pathologies with a known inflammatory component, such as multiple sclerosis (MS), miRNAs have also been shown to play a central role in the regulation of microglia-mediated immune responses. Ponomarev and coworkers have demonstrated that miR-124 is able to switch microglia from an inflammatory to a quiescent state, and this phenomena was considered essential to successfully inhibit the onset of EAE [33]. Also in MS, miR-155$^{-/-}$ knockdown mice were shown to present significantly reduced numbers of encephalogenic CD4^{+} Th17 cells, an inflammatory T-cell subset with an important role in this disease [70]. In a very recent study, Butovsky et al. showed that the modulation of the cytokine profile of inflammatory monocytes using an anti-Ly6C mAb reduced monocyte recruitment to the spinal cord, decreased neuronal loss, and extended survival in a mouse model of ALS. Interestingly, in humans with ALS, the analogous CD14^{+}CD16^{-} monocytes were shown to exhibit an ALS-specific miRNA inflammatory signature, which can be used as a biomarker of this disease and reveal novel therapeutic targets [71].

Another interesting effect associated with miRNAs derives from their capacity to activate receptors by themselves, after being secreted by cells within the CNS, which allows them to function as signaling molecules. For example, let-7 is known to induce neurodegeneration by binding to TLR7 in neurons and microglia. Let-7 is increased in the CSF of AD patients, and accordingly, the injection of this miRNA in the CSF of wild-type animals caused neurodegeneration, which did not occur in mice lacking TLR7 [72]. Also in the context of AD, the NF-κB-dependent miR-146a was reported to be upregulated in the brain of AD patients and to enhance inflammation by targeting the complement factor H (CFH) [49]. MiR-146a was also found to be overexpressed in prion-infected mouse brain tissues, concurrent with the onset of prion deposition and microglia activation, which suggests that this miRNA plays a role in the proinflammatory response of microglia to prion replication [73].

The above-mentioned studies stress the role of miRNAs as modulators of both neuroinflammation and neurodegeneration and illustrate their potential as biomarkers and novel therapeutic targets in CNS diseases.

6. MiRNA-Based Therapeutic Applications in Neuroimmune and Neurodegenerative Diseases

Over the last few years, there has been a significant progress in the development of strategies to modulate the levels of certain miRNAs and miRNA clusters, aiming at adjusting cellular functions dysregulated in several pathologies. Modulation of mature miRNAs can be accomplished by oligonucleotides complementary to miRNA sequences (miRNA inhibitors), causing miRNA knockdown or, alternatively, by miRNA precursor oligonucleotides (miRNA mimics), which cause miRNA overexpression [74–76]. Usually, these oligonucleotides present chemical modifications, such as the incorporation of locked nucleic acids (LNA) nucleotides, which have a methylene bridge between the 2′-oxygen and the 4′-carbon of the ribose moiety or the incorporation of 2′-O-methyl modified nucleotides (antagomirs) in certain positions of the oligonucleotide sequence. The purpose of these modifications is to increase the chemical stability and resistance of the miRNA inhibitors or mimics to serum nucleases, thus potentiating their therapeutic potential. However, in order to achieve a successful clinical application of miRNA-based therapies it is crucial that these oligonucleotides are properly delivered by vehicles that not only reliably and effectively overcome cellular and physiological barriers but are also highly target specific. The presence of the BBB, which restricts entry of therapeutic molecules into the brain, as well as the possible degradation of nucleic acids by nucleases present in the blood, constitutes major obstacles associated with nucleic acid delivery *in vivo*. Nonviral vectors have been developed to ensure protection and improvement of nucleic acid delivery into target cells and have been employed in miRNA-based therapeutic approaches to modulate neuroinflammatory signaling pathways [77].

In our own work, we have shown that the use of a nonviral strategy to promote miR-155 silencing in microglia, prior to their activation, is able to reduce the release of NO and other inflammatory mediators, such as the major inflammatory cytokines TNF-α and IL-6 to the extracellular environment, through an increase in the levels of SOCS-1, a direct target of miR-155 (Figure 2). The modulation of this miRNA in microglia cells, prior to their activation with LPS, proved to be enough to improve cell viability in neuronal cultures incubated with microglia conditioned medium [18]. These results stress the neuroprotective potential of miR-155 silencing in the context of neuroinflammation.

Other miRNAs have been related with the regulation of the neuroimmune response. As discussed before, miR-124 is one of the main miRNAs responsible for the maintenance of the quiescent state of microglia/macrophages in the brain and spinal cord, and therefore, miR-124 may constitute an important target for the development of therapeutic approaches towards the control of neuroinflammation. Recently, Willemen and coworkers investigated the contribution of miR-124 to the regulation of hyperalgesia and microglia/macrophage activation in LysM-GRK2$^{+/-}$ mice, in which the expression of the G protein-coupled receptor kinase (GRK) 2 is decreased to about 50% in microglia/macrophages, with respect to wild-type animals. These mice develop inflammatory hyperalgesia caused by activation of microglia/macrophages in the spinal cord. The authors showed that the pathological transition from acute to persistent hyperalgesia is associated with reduced levels of miR-124 in spinal cord microglia and with a microglia M1 phenotype switch, leading to increased production of proinflammatory cytokines. The intrathecal administration of miR-124 mimics prevented completely the transition to persistent pain in LysM-GRK2$^{+/-}$ mice and normalized the expression of microglial M1/M2 markers, suggesting that miR-124 administration may be a promising approach to prevent and treat persistent inflammatory and neuropathic pain [78].

Another relevant study on miRNA therapeutics in the context of neuroinflammation was reported by Selvamani

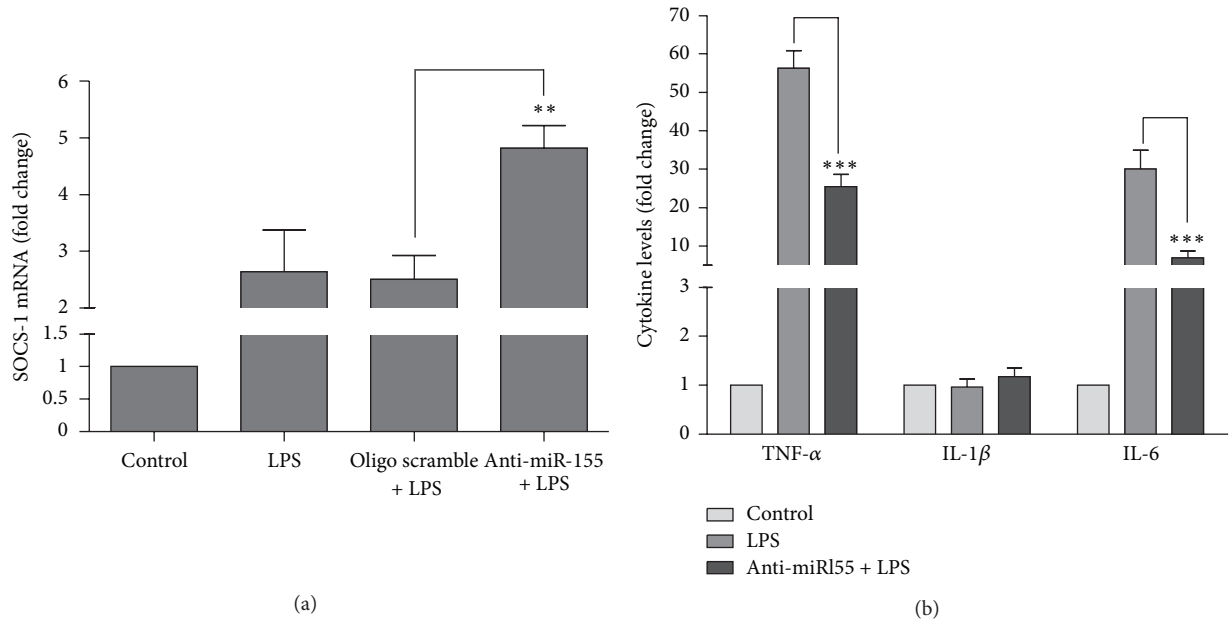

Figure 2: MiR-155 inhibition in microglia cells increases SOCS-1 levels and decreases the release of inflammatory cytokines to the extracellular medium. N9 mouse microglia cells were transfected with anti-miR-155 oligonucleotides (anti-miR155) or with nontargeting oligonucleotides (oligo scramble) complexed with cationic liposomes for 4 h. Twenty-four hours after transfection, cells were incubated with LPS at 0.1 μg/mL for 18 h. The cell culture medium was then collected to determine cytokine protein levels, and total RNA was extracted from the cultured cells. (a) SOCS-1 mRNA levels were quantified by qRT-PCR. (b) The levels of TNF-α, IL-1β, and IL-6 secreted to the cultured medium were determined by ELISA. Results are expressed as fold change of mRNA or protein levels with respect to control (untransfected and untreated cells). $^{**}P < 0.01$ compared to cells transfected with the scramble oligonucleotide and $^{***}P < 0.01$ compared to LPS-treated cells in the absence of transfection. Results in (a) and (b) are representative of 3 independent experiments performed in triplicate.

and coworkers. The authors hypothesized that miRNAs able to target proteins from the insulin-like growth factor-1 (IGF-1) signaling family could be suppressed to promote the neuroprotection provided by IGF-1 following stroke. Using middle-age female rats in which the treatment with estrogen is known to be neurotoxic, the authors administered stereotactically LNA antisense oligonucleotides against miR-1 or let-7f, 4 h after stroke, and observed that miRNA inhibition extended the neuroprotection afforded by IGF-1. Interestingly, although let-7f is a proinflammatory miRNA preferentially expressed in microglia from the ischemic hemisphere, where the IGF-1 expression is detectable, the levels of IGF-1 increased even further in microglia after anti-let-7f treatment [79]. Finally, a study from Lukiw and coworkers showed that the expression of miR-146a is increased in AD brains, which was correlated with a decrease in the CFH expression, a protein responsible for the repression of the inflammatory response in the brain. The inhibition of miR-146, achieved through delivery of a specific LNA-antisense oligonucleotide in human neural cells subjected to oxidative stress, was found to restore CFH expression levels, which were decreased following oxidative damage [49].

Taken together, these reports illustrate the vast neuroprotective potential of miRNA-based therapeutic strategies using anti-miRNA oligonucleotides targeting neuroinflammation and confirm the important role of miRNAs in modulating neuroimmune responses to acute and chronic brain damage. Although less explored, the application of miRNA mimics, in order to restore miRNA expression and decrease the levels of potentially neurotoxic proteins, is also an interesting possibility with high therapeutic potential. Nevertheless, it is important to mention that, due to incomplete pairing, each miRNA has the ability to target multiple genes simultaneously, which significantly increases the risks of unspecific effects on miRNA-based therapeutic approaches. To avoid this drawback, it will be relevant to consider not only the strength of the binding between a certain miRNA and all its available targets in a specific tissue but also the relative amounts of these targets and the thermodynamic stability of each miRNA : mRNA duplex [80].

7. Conclusion

Although the exact nature of the contribution of microglia and peripheral mononuclear phagocytes to neurodegeneration remains to be fully elucidated, several benefits have been suggested for the use of these immune cells in therapeutic strategies designed to curb amyloidosis, decrease EAE progression, fight CNS viral infection, or assist in the reduction of neuroinflammation associated with neurodegenerative diseases. On the other hand, several groups have identified signs of chronic activation and functional impairment in microglia cells isolated from different brain disease models. Given their important role in the regulation of gene expression, we believe that miRNA-based therapies could constitute an interesting and attractive strategy to improve

microglia activity, modulating signaling pathways linked with neuroinflammation. In addition, the compromised activity of the BBB in most neurodegenerative disorders, the lower activation threshold of peripheral mononuclear phagocytes compared to brain-derived microglia, and their easy access in a clinical context make these cells another excellent tool for future gene therapy approaches for brain disorders. Overall, understanding the contribution of brain- and blood-derived immune cells to microglia pools in the CNS in a disease context will be of great importance to improve the existing immunotherapies and generate new and effective therapeutic strategies for these diseases.

The fine-tuning activity of miRNAs has been proven crucial in the regulation of differentiation of microglia allowing the maintenance of brain homeostasis. Since a single miRNA has the capacity to target more than one protein involved in the same signaling pathway, their modulation can significantly change cell phenotypes that depend on the levels and activation of specific proteins. Such capacity reflects a molecular paradigm suitable for therapeutic intervention. Due to the lack of minimally invasive diagnosis tools and effective therapeutic options for most CNS diseases, we believe that the use of miRNAs, both as disease biomarkers and therapeutic targets associated with cells of the immune lineage, although yet poorly explored, will tend to grow in the near future.

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Review Article

Microglial Dysregulation in Psychiatric Disease

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Microglia, the brain's resident immune cells, are phagocytes of the macrophage lineage that have a key role in responding to inflammation and immune challenge in the brain. More recently, they have been shown to have a number of important roles beyond immune surveillance and response, including synaptic pruning during development and the support of adult neurogenesis. Microglial abnormalities have been found in several neuropsychiatric conditions, though in most cases it remains unclear whether these are causative or are a reaction to some other underlying pathophysiology. Here we summarize postmortem, animal, neuroimaging, and other evidence for microglial pathology in major depression, schizophrenia, autism, obsessive-compulsive disorder, and Tourette syndrome. We identify gaps in the existing literature and important areas for future research. If microglial pathology proves to be an important causative factor in these or other neuropsychiatric diseases, modulators of microglial function may represent a novel therapeutic strategy.

1. Introduction

Microglia are the brain's resident immune cells. Unlike neurons and other types of glia, which are of neuroectodermal origin, microglia are macrophage-lineage cells derived from hematopoietic progenitors. Convergent data suggests that microglial activation occurs in a number of neuropsychiatric conditions. This raises intriguing questions about the contribution of dysregulated brain-immune interactions to the pathogenesis of these conditions. Here we review the clinical and preclinical literature implicating microglia in the pathophysiology of several psychiatric disorders.

Historically, microglia have been presumed to be quiescent under physiological conditions and activated only upon immune challenge or in response to neuronal damage or debris. This is consistent with a role in neurodegeneration, in which microglial activation could be a consequence of a degenerative process, as these phagocytic cells participate in cleaning up cellular debris. Alternatively, dysregulated activation of cytotoxic microglial processes may contribute to neuronal damage and degeneration. A role for microglial activation has long been suggested in the pathophysiology of neurodegenerative conditions such as Alzheimer's disease, Parkinson's disease, and dementia associated with the human immunodeficiency virus.

More recently, a series of important findings have challenged the notion that microglia are dormant when not activated by inflammation or immune challenge. Microglia have been found to be required for the development of mature synapses during embryogenesis [1] and to regulate the number of functional synapses both in culture [2] and *in vivo* [3]. They also regulate adult neurogenesis [4]. This new appreciation that microglia regulate neuronal function and homeostasis under physiological conditions, in the absence of immune challenge or inflammation, raises the possibility that disruption of such processes may contribute to pathological conditions characterized by neuronal or synaptic dysfunction, rather than frank neurodegeneration. In particular, dysregulated synaptic physiology has been a major focus of recent interest in studies of the pathophysiology of mood [5] and psychotic disorders [6], and abnormalities of neurogenesis may similarly contribute to psychiatric disease [7].

The observation of microglial activation or other abnormalities in any particular psychiatric condition does not establish whether microglia are causal contributors to the pathophysiological process or, rather, are activated in response to cellular damage or other aspects of the core pathology. Experimental studies recapitulating pathophysiological processes in animal models provide the best avenue to explore this challenging question of causality. Such investigations are in their infancy, and a pathogenic role for dysregulated microglia in any neuropsychiatric condition remains largely hypothetical. This is an exciting area of ongoing research in neuropsychiatry.

2. Microglia

An exhaustive discussion of microglial physiology is beyond our scope here. Before summarizing evidence for a contribution of microglial dysregulation to several psychiatric conditions, we briefly introduce key points and molecular markers that are specifically relevant to that discussion.

Microglia are small cells of the macrophage lineage found throughout the brain. They are readily identified in brain tissue by their expression of a variety of macrophage markers; several of these have been widely used in the studies summarized below and merit specific mention (for a review see [8]). Microglia, like macrophages, can be identified by their expression of the marker CD11b (also known as complement receptor 3). The expression of the ionized calcium-binding adapter molecule 1 (Iba1) is restricted to microglial cells and is an excellent marker for the analysis of microglial ramifications (Figure 1). The activation of microglia, and of peripheral macrophages that infiltrate the brain under pathological conditions, can be monitored by their expression of CD45; resting microglia are $CD45^{low}$, whereas macrophages are $CD45^{high}$. These two populations can be readily distinguished and isolated by flow cytometry. Microglial activation also leads to upregulation of CD11b and Iba1. Microglia also express macrosialin (CD68), a molecule involved in phagocytosis.

Microglia take several morphological forms. Early in development they have an amoeboid appearance, similar to peripheral macrophages, while in the adult central nervous system they take on characteristic ramified morphology, with long, thin processes (Figure 1). Microglia are highly motile cells, extruding and retracting their processes every few minutes [9]. This has been interpreted as an active sampling of the environment; given the number of microglia in the brain and their motility, it has been calculated that they can explore the entire extracellular space in the brain in a few hours [10]. Such extensive sampling may allow them to search for signs of infection, cellular debris, or other inducing stimuli.

Exposure to bacterial antigens such as lipopolysaccharide (LPS) produces the classical cytotoxic activated microglial phenotype. Activated microglia may become hyperramified or amoeboid/phagocytic [11]. Hyperramified microglia exhibit increased arborization, with thick processes. During the transformation into the amoeboid form, microglia retract the processes and enlarge their cell bodies. Activated microglia produce the proinflammatory cytokines interleukin (IL)-1β, tumor necrosis factor (TNF)-α and IL-6, among others [12]. LPS-activated microglia also upregulate the inducible form of the nitric oxide synthase (iNOS) and produce nitric oxide. This activation of microglial cells is required for their effector immune function in the normal brain. However, dysregulation of this physiological process can lead to neurodegeneration [8, 13].

Another molecular signature of microglia activation is the expression of the major histocompatibility complex (MHC) class II, or human leukocyte antigen (HLA-DR, -DP, and -DQ), which serves as an antigen presenter to T helper cells ($CD3^+CD4^+$). $CD25^+$ T helper cells (regulatory T cells, or Tregs) are particularly important to the biology of microglia, and the interaction between these cell types has been implicated in the pathophysiology of neurodegenerative diseases. For example, in mouse models of Parkinson disease and HIV infection-associated neurodegeneration, Tregs were found to have a neuroprotective effect, attenuating microglia-mediated inflammation [14, 15]. $CD25^-$ effector T cells (Teffs) were found to have the opposite effect [15]. It has been hypothesized that protective Tregs recognize self-antigens and mediate protective autoimmunity [16, 17].

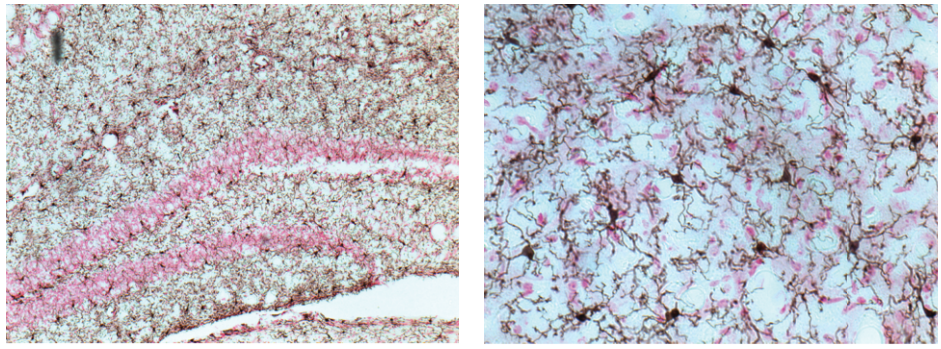
Microglia can also adopt a neuroprotective phenotype upon activation by cytokines such as IL-4 or IL-25 [18, 19]. These neuroprotective microglia do not produce neurotoxic cytokines like TNF-α. Rather, they produce insulin-like growth factor (IGF)-I and transforming growth factor (TGF)-β, among others [18, 20].

As noted above, microglia are of the hematopoetic lineage, though they populate the brain early in development. Under conditions of inflammation, additional macrophage-lineage cells can be recruited into the central nervous system and differentiate into a microglia-like phenotype. Activated microglia produce high levels of the chemokine (C-C) motif ligand 2 (CCL-2), also known as monocyte chemotactic protein-1 (MCP-1). CCL2/MCP-1 triggers microglia proliferation and also serves as a signal for microglia-induced neurodegeneration [21, 22]. As suggested by its name, MCP-1 also acts a recruiter of other inflammatory cells to the brain. Unlike resident microglial cells, infiltrating macrophages express the CCL2 receptor (CCR2) at high levels.

Fractalkine (CX3CL1) and its receptor (CX3CR1) are also involved in immune cell trafficking to the central nervous system [23]. CX3CR1 expression, unlike that of CCR2, is restricted to microglia in the brain. Mice that lack the CX3CR1 have impaired cognitive function and synaptic plasticity [24]. CX3CR1 has also been implicated in the physiological synaptic pruning mediated by microglia, a process that is needed for normal development of neural circuits; knockout animals have increased dendritic spines and immature synapses [1]. CX3CR1+ microglial cells are also required to support hippocampal neurogenesis [25].

3. Microglial Dysregulation in Depression and Anxiety Disorders

A link between immune dysregulation and the pathophysiology of at least some forms of major affective disorder has long



(a) (b)

Figure 1: (a) Distribution of Iba1+ microglial cells in the mouse hippocampus. Total cells are stained with fast red. (b) High magnification of microglial staining in the striatum, showing cell bodies and ramifications.

been hypothesized, on the basis of several observations. Many studies have shown abnormalities in peripheral cytokines in depressed patients; indeed, these data have led some investigators to propose a primary immunological etiology for major depressive disorder [7]. Certain core symptoms of major depression, especially the somatic symptoms, resemble the "sickness behavior" that is produced by systemic infectious or inflammatory conditions [26]. Furthermore, significant depressive symptoms are frequently seen following treatment with the cytokine interferon alpha in the context of hepatitis C [27].

As the primary resident immune cells in the brain, microglia are obvious candidate mediators of abnormal brain-immune dialogue in depression. However, clinical evidence implicating microglial dysregulation in affective disorders is limited. In a postmortem study of frontal cortex in several neuropsychiatric conditions, Bayer et al. [28] found increased hippocampal microglia activation (as visualized by HLA-DR expression) in only one of 6 patients with major affective disorders. Similarly, Steiner et al. [29] analyzed several brain regions (dorsolateral prefrontal cortex, anterior cingulate cortex, mediodorsal thalamus, and hippocampus) in postmortem samples from depressive patients and did not find alterations of microglial density. Another report examined CD11b mRNA expression and found no differences in patients with mood disorders (major depression and bipolar disorder) compared to controls [30]. However, significant microgliosis has been observed in patients with depression who completed suicide, compared to patients who died via other methods and healthy controls [29].

Furthermore, a study comparing depressed patients who completed suicide to healthy controls demonstrated an increased density of microglia positive for quinolinic acid, an N-methyl-D-aspartate (NMDA) glutamate receptor agonist produced and released by activated microglia and by no other cells in the brain [31]. Abnormalities in glutamatergic neurotransmission have been implicated in depression by recent studies [32, 33], and glutamate modulators have been proposed as novel antidepressant agents [33, 34]. At sufficient doses, quinolinic acid (QA) is a neurotoxin, a gliotoxin, a proinflammatory mediator, and an oxidant and can alter the integrity and cohesion of the blood-brain barrier [35]. All of these effects—inflammation, oxidative stress, impaired neurogenesis, reduced glial cell number, and neuronal damage—have been implicated in depression [36]. Whether QA produced by activated microglia contributes to these phenomena under physiological conditions in the pathogenesis of depression remains an open question.

Animal models of mood disorders have been used to further investigate the potential pathogenic role of microglia. Chronic psychological stress, which can contribute to the development of depression [37], increases microglia activation in the prefrontal cortex of rats; the antibiotic minocycline, an anti-inflammatory drug that blocks microglial activation, is able to reverse both microglial abnormalities and attendant cognitive dysfunction in stressed animals [38]. Interestingly, minocycline also produces antidepressant-like effects in rats subjected to learned helplessness, a model of depression [39]. Another chronic stress model, repeated social defeat, increased the presence of deramified Iba1$^+$ microglia in the medial amygdala, prefrontal cortex, and hippocampus, with increased levels of cytokines associated with cytotoxic microglial activation—IL-1β, IL-6, TNF-α, and iNOS—in CD11b$^+$ cells [40, 41].

Conversely, events that activate microglia can have long-lasting behavioral consequences. Neonatal exposure of rats to LPS produces significantly increased anxiety-like behavior and hippocampal microglial activation in adulthood [42, 43].

Further animal evidence for a pathogenic role for microglia derives from mice deficient in the fractalkine receptor, CX3CR1, whose expression in the brain is restricted to microglia. These mice displayed enhanced depression-like behavior after treatment with LPS, which also triggered a persistent activated microglial phenotype in the hippocampus and prefrontal cortex [44]. Purified CD11b$^+$/CD45^{low} microglia from knockout mice expressed higher levels of IL-1β than wild-type controls after LPS challenge [8]. These results suggest that CX3CR1/CX3CL1 negatively regulates depressogenic actions of activated microglia [44], perhaps by directing microglia towards a neuroprotective phenotype [18, 19]. Interestingly, two enzymes in the quinolinic acid biosynthesis pathway—indoleamine 2,3-dioxygenase

(IDO) and kynurenine monooxygenase (KMO)—were also increased in microglia from CX3CR1 knockout mice [44]. Pharmacological inhibition of IDO counteracted the LPS-induced depressive-like state in CX3CR1 knockout mice [45], providing evidence for the functional importance of QA in microglia-mediated pathogenic effects.

Several antidepressants have been found to prevent the neurodegenerative activation of microglia induced by LPS and cytokines *in vitro* [46–52]. This effect has been seen with different classes of antidepressants, including selective serotonin reuptake inhibitors, selective norepinephrine reuptake inhibitors, tricyclic antidepressants, and even ketamine. An exception is the monoamine oxidase inhibitor phenelzine, which was found to synergize with LPS in activating microglia, albeit at high concentrations [53].

4. Microglial Abnormalities in Schizophrenia

Several lines of evidence suggest immune dysregulation in the pathogenesis of schizophrenia. For example, maternal infection during pregnancy has been associated with schizophrenia, at least at the epidemiological level (reviewed in [54, 55]).

A small early postmortem study found aberrant activation of microglial cells characterized by HLA-DR expression in a subset of individuals with schizophrenia [28], though other small early studies did not replicate this finding [56]. More recently, morphological analysis in postmortem tissue found evidence both of microglial activation and of microglial degeneration. A pair of larger postmortem studies found evidence of degeneration in HLA+ microglia cells from schizophrenic patients, such as cytoplasm shrinkage, damaged mitochondria, thinning, and shortening and fragmentation of their processes [57, 58]. Ultrastructural analysis has revealed activation of pericapillary microglia with enlarged and vacuolated cytoplasms, irregular nuclear contours, and increased lysosomes [59]. In another recent study, the density of cells positive for the β subunit of the MHC-II, which is common to HLA-DP/DR/DQ and is expressed on activated microglia, correlated with IL-1β expression in the brains of schizophrenic patients [60]. Although activated microglia are not the only possible source of IL-1β, the significant statistical correlation with the microglia-specific marker MHC-II/HLA suggests that they are likely to be the source in these brains.

Several other postmortem studies have provided further evidence of microglial activation, and of brain infiltration by other immune cells, in schizophrenia [29, 61–64]. One of these found differential microglial activation in different clinical subtypes of schizophrenia; HLA-DR$^+$ microglia were increased in the posterior hippocampus of individuals with paranoid schizophrenia relative to those with residual schizophrenia. In contrast, patients with residual schizophrenia had a greater density of CD3$^+$ and CD20$^+$ lymphocytes in the same region [63].

Recently it has become possible to quantify microglial activation *in vivo* using a positron emission tomography (PET) ligand that recognizes the translocator protein (TSPO), a receptor found on activated microglial cells (as well as on other peripheral cell types). Binding of one such agent, (*R*)-[^{11}C]PK11195, was increased, suggesting differential microglial activation, in gray matter [65] and in hippocampus [66] of patients with schizophrenia.

Animal models of schizophrenia face vexing challenges to their validity; with that caveat, findings in a few models support a possible role for microglial dysregulation. An animal model based on a cryolesion in the parietal cortex of juvenile mice, which produces later cortical atrophy and cognitive decline reminiscent of that observed in schizophrenia, induces a lasting increase in the number of microglia in cingulate cortex and hippocampus, with accompanying neurodegeneration [67]. An increased number of microglial cells and reduced arborization, which suggests activation, were also found in the hippocampus and the striatum of young rodents following embryonic polyriboinosinic-polyribocytidylic acid (Poly I:C) exposure [68, 69], which is proposed to recapitulate disrupted brain-immune interactions associated with schizophrenia and autism. Similar microglial abnormalities were observed in an experimental model of schizophrenia associated with hyperbilirubinemia [70].

Other findings are consistent with increased microglial activation in schizophrenia having a pathogenic role, and with its modulation having a role in treatment. The gene DISC-1 (disrupted-in-schizophrenia-1), in which mutations have been associated with schizophrenia and other serious mental illnesses in a large pedigree, is expressed in CD11b$^+$ microglia, as well as in neurons [71]. *In vitro* studies have shown that several antipsychotics, including olanzapine, risperidone, aripiprazole, spiperone, perospirone, and ziprasidone, can inhibit microglia activation [72–77].

If microglial activation contributes to the pathophysiology of schizophrenia, then direct modulators of microglia function may be effective in the treatment of psychotic disease. The antibiotic and anti-inflammatory drug minocycline reduces microglial activation. A few years ago, uncontrolled case series began to appear reporting therapeutic benefit from the addition of minocycline to antipsychotic treatment in schizophrenia [78, 79]. In one subject treated with eight weeks of minocycline, added to stable antipsychotic treatment, perfusion of the posterior cingulate cortex was reduced after the minocycline augmentation [80]. In open-label studies, Miyaoka and colleagues found a significant decrease in both positive and negative symptoms after minocycline was added to an antipsychotic in subjects with schizophrenia [81], and when it was added to an antipsychotic and antidepressant in individuals with psychotic depression [82].

More recently, adjunctive minocycline, added to stable antipsychotic treatment, has been examined in controlled clinical trials, with promising early results. Two double-blind, placebo-controlled studies showed a beneficial effect of adjunctive minocycline therapy on the negative symptoms of schizophrenia [83, 84] and on cognitive function [83], compared to subjects receiving standard antipsychotic therapy. In all reports, the addition of minocycline to the treatment regimen was well tolerated. These reports provide a first therapeutic application of the theory that microglial activation may contribute to psychotic illnesses.

5. Microglia in Autism and Rett Syndrome

Several *postmortem* studies have suggested a role for microglial pathology in autism spectrum disorders. An initial study found marked microglial activation, measured by immunohistochemical quantification of HLA-DR expression, in the cerebellum, several cortical regions, and white matter in patients with autism [85]. A subsequent study described both increased density of microglial cells in the dorsolateral prefrontal cortex and a shift towards an amoeboid morphology, characterized by soma enlargement, process retraction and thickening, and extension of filopodia from processes, that is suggestive of activation and differentiation into the cytotoxic phenotype [86]. Similar results have been reported in the frontoinsular and visual cortices [87]. Interestingly, the organization of microglia-neuron interactions may be abnormal in autism; microglia are distributed closer to neurons of the dorsolateral prefrontal cortex [88]. The encirclement of neurons by microglial processes suggests an important role of this cell-cell interaction in the pathophysiology of autism.

These *postmortem* findings have been supported more recently by PET imaging with [^{11}C]PK11195, the microglia-binding ligand described above. Increased [^{11}C]PK11195 binding was observed in multiple brain regions (cerebellum, midbrain, pons, fusiform gyri, and the anterior cingulate and orbitofrontal cortices) in young adult subjects with autism spectrum disorder, suggesting increased microglial activation [89]. These PET findings must be interpreted with caution, as there is no microglia-free reference region to which binding can be normalized, but in conjunction with the postmortem work they build a consistent case for microglial excess in at least some cases of autism.

Similar abnormalities have been reported in several animal models that recapitulate aspects of the pathophysiology or symptomatology of autism. For instance, BTBR *T*+*tf*/J mice, which exhibit reduced social interaction and a restricted behavioral repertoire, recapitulating some of the core symptoms of autism, have increased MHC2-expressing microglia compared to control mice [90]. Terbutaline, a β2-adrenoceptor agonist used to arrest preterm labor, has been associated with increased concordance for autism in dizygotic twins [91]; postnatal administration of terbutaline to rat pups resulted in microglial activation and behavioral abnormalities that resemble apsects of autism [92]. Propionic acid-induced autistic-like behavior in laboratory animals is also accompanied by microglial activation, assayed as increased CD68 expression [93, 94].

Rett syndrome, an X-linked autism spectrum disorder characterized by the mutation of the methyl-CpG-binding protein-2 (MECP2) gene, has recently been associated with microglial dysfunction. MECP2-deficient microglia release excess glutamate, *in vitro*, via connexin 32 (Cx32) hemichannel-mediated release, as a consequence of enhanced glutaminase expression [95]. Interestingly, increased levels of glutamate and glutamine, measured using magnetic resonance spectroscopy (MRS), have been reported in young patients with Rett syndrome [96], suggesting that a similar glutamate dysregulation may occur *in vivo* in patients. Reductions in AMPA and NMDA glutamate receptor density in the putamen and in kainate (KA) glutamate receptor density in the caudate of Rett patients have also reported [97]. Similarly, glutamatergic neurotransmission is impaired in the animal model of this disease [98]. Immune-mediated dysregulation of glutamatergic neurotransmission has been proposed as a pathogenic mechanism in autism spectrum disorders more generally [99].

Most of the associations catalogued above between microglial dysregulation and psychopathology are correlational. A groundbreaking recent study in an animal model of Rett syndrome provides one of the few clear pieces of evidence for the causal importance of such an association. Restoration of wild-type microglia by bone marrow transplantation or genetic rescue attenuated the Rett syndrome-like symptomatology in MECP2-null mice [100]. This immediately suggests therapeutic possibilities in this devastating disease.

6. Microglia in Obsessive-Compulsive Disorder and Tourette Syndrome

Several lines of evidence have long suggested an association between immune dysregulation and obsessive-compulsive disorder (OCD) (e.g., [101, 102]). A specific role for microglia in the pathophysiology of OCD, or of the related compulsive grooming disorder, trichotillomania, has been suggested by a recent study in a mouse model. Ten years ago, mice with a knockout of the HoxB8 gene, a homeobox developmental patterning gene expressed prominently in macrophage-lineage hematopoietic cells, were observed to exhibit excessive grooming behavior; this excessive grooming has been proposed to model OCD symptomatology [103] More recently, HoxB8 mutant microglia were found to be necessary and sufficient for this excessive grooming phenotype. The phenotype can be rescued by transplantation of wild-type bone marrow into mutant Hoxb8 mice, which leads to repopulation of the brain with wild-type microglia. Conversely, transplant of Hoxb8 mutant bone marrow into wild-type mice can induce the pathological grooming behavior [104]. The mechanisms of this fascinating effect remain unclear. In the nervous system, Hoxb8 plays an important role in the formation of the spinal cord, sensory responses, and development of noradrenergic autonomic neurons [105, 106]. In the immune system, Hoxb8 appears to be involved in the maintenance of monocyte precursors by blocking differentiation of myeloid progenitors from primary marrow into macrophages, dendritic cells, and probably microglia [107–109]. In the brain, a subset of microglia—not all—express HoxB8. The specific physiological role of this particular subset of Hoxb8$^+$ microglia has yet to be described.

There have been few postmortem studies in OCD, and none described to date have investigated microglial activation. Therefore, while these observations in HoxB8 knockout mice are fascinating and suggest exciting new directions for research, their direct applicability to the pathophysiology of OCD remains to be established. No mutations in the HoxB8

gene have yet been described in association with OCD, grooming disorders, or related conditions.

Tourette syndrome is a developmental neuropsychiatric disorder characterized by involuntary motor and phonic tics; it has a high comorbidity with OCD. There is as yet no direct *postmortem* evidence for microglial dysregulation in Tourette syndrome, but several molecular findings suggest a possible relationship. A nonsignificant two-fold increase in the expression of the surface marker CD45, which is higher in activated than resting microglia, was reported in postmortem basal ganglia [110]. Additionally, elevated expression of CCL2/MCP-1 was observed in these patients. Elevation of this chemokine may promote microglial activation, particularly of the subtype that express its receptor CCR2 [111]. The involvement of microglial dysregulation in Tourette syndrome is an intriguing area for future study.

7. Conclusion: Open Questions and New Directions

The data summarized above provide intriguing evidence for microglial dysregulation in a number of psychiatric conditions. However, they also highlight several important areas for ongoing research before these associations can be considered conclusive.

First, the direct data for associations between microglia and psychopathology rest in many cases on small *postmortem* studies; in the case of OCD, direct human data are entirely lacking. Ongoing high-quality *postmortem* work is essential to strengthen the evidence that microglial abnormalities are seen in these conditions. Findings in animal model systems are intriguing and can provide important supportive data and mechanistic insight, but because of the difficulties in fully modeling the pathophysiology of psychiatric disease in an animal, they cannot substitute for direct observations in human tissue. The recent development of PET ligands that can measure microglial activation, such as [^{11}C]PK11195, provides an important new source of parallel data for microglial activation in patients.

Second, in many cases, *postmortem* investigations have revealed abnormal microglial activation in only a subset of individuals. For example, in one early investigation across several disorders [28], excessive microgliosis was seen in one of six subjects with major affective disorders and three of 14 subjects with schizophrenia. This suggests that, to the extent that abnormal microglial activation contributes to disease, it does so in a heterogeneous fashion. The distinction between patterns of immune dysregulation in paranoid versus residual schizophrenia described above [63] provides one candidate hypothesis to explain this heterogeneity. It will be important to better characterize which patients within each heterogeneous clinical population exhibit microglial abnormalities. PET imaging of microglial activation is likely to be an essential tool for this project. A related question is how microglial activation differs between phenotypically distinct disorders. If microglia are excessively activated in both depression and schizophrenia, what determines the difference between these conditions?

Third—and perhaps most importantly—the causal role of microglial activation in the pathophysiology of these conditions remains to be established. Most of the data described above are purely correlational and leave open the question of whether microglial pathology is a cause of neuronal dysfunction and damage and of behavioral symptomatology or, rather, whether neurons (and/or glia) are damaged by independent pathological processes and microglial activation develops as a consequence. In this regard, the recent demonstration that bone marrow transplant (and thus microglial replacement) can mitigate core phenotypes in a Rett model mouse is of immense importance [100]. This represents the best evidence for a causal role, rather than a reactive one, for microglial pathology in the development of behavioral symptomatology. Similar evidence in the HoxB8 knockout mouse [104] establishes a parallel causal role for microglia in their grooming phenotype, but its clinical importance is lessened by the still-tenuous connections between excessive grooming in this mouse model and the pathophysiology of OCD in human patients. The apparent ability of minocycline augmentation to improve the symptoms of schizophrenia [83, 84] provides some early clinical evidence for a causal role for microglial activation in this condition, though this conclusion is limited by minocycline's multiple mechanisms of action.

Finally, as has been emphasized at several points above, microglia can take on distinct phenotypes, and the separate contribution of these different subsets of microglial to psychiatry pathophysiology remains almost entirely obscure. For example, different cytokines can lead microglia to differentiate into cytotoxic or neuroprotective phenotypes; how these are differently dysregulated in various psychiatric conditions remains to be demonstrated. Similarly, only a subset of microglia in a wild-type mouse express the HoxB8 gene; the functional importance of this subset is emphasized by the phenotype of the HoxB8 knockout mouse, but the mechanistic details remain unclear. Finally, there is growing appreciation of the distinction between brain resident microglia and macrophage-lineage cells that enter the brain later during development, or even in adulthood [111, 112]. Functional distinctions between these microglial subpopulations are not yet well understood.

Our understanding of the role of microglia in normal brain function is rapidly evolving. Until recently, these resident immune cells were thought to be entirely passive in the absence of an immune challenge and to become activated only in the context of inflammation. Exciting recent data have revealed important role for microglia even in the absence of any inflammation or immune challenge. This is particularly clear in the context of brain development; the role for microglia in normal adult brain function and homeostasis is less well established.

These recently appreciated noninflammatory functions of microglia create a rich new field for the understanding of pathophysiological processes. The evidence for microglial dysregulation in the pathophysiology of several psychiatric conditions, which we have summarized here, is intriguing. While these associations remain inconclusive in most cases, this is an exciting area of ongoing research. To the extent that

microglial dysregulation proves to be causally important in the development of neuropsychiatric disease, it may provide a fruitful new area for therapeutic intervention.

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