# Mediators of Neuroinflammation

Guest Editors: Geeta Ramesh, Mario T. Philipp, Luc Vallières, Andrew G. MacLean, and Muzamil Ahmad

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Editorial Board

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Editorial
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The understanding that neuroinflammation contributes to neurodegeneration and neuropathic pain is an emerging feature in a growing number of nervous system pathologies. Despite the immunoprivileged status of the central nervous system (CNS), its resident macrophages, microglia, with the help of other immune cells recruited from the blood, can mount robust attacks against intraparenchymal targets. Microglia also contributes to the repair of the CNS after injury and eliminates toxic molecules produced during degenerative diseases, but only modestly, possibly due to physiological adaptations that limit inflammation and its potentially devastating side effects in the CNS. Further, the complex networks of nerve cells, supportive and regulatory glial cells such as astrocytes, and oligodendrocytes in the brain and spinal cord, Schwann cells in peripheral nerves and satellite glial cells in the dorsal root ganglia as well as other cells like endothelial cells, can secrete immunoregulatory factors capable of mediating neuroinflammation.

Although some inflammatory stimuli induce beneficial effects that help to limit disease, for instance, the killing of infectious microorganisms and elimination of damaged cells, uncontrolled inflammation may result in the production of neurotoxic factors that amplify underlying disease states. In addition, in some neuroinflammatory diseases like multiple sclerosis (MS), a breakdown of tolerance to self-antigens occurs by some unknown mechanism, leading immune cells to degrade the myelin sheath that surrounds axons. In contrast, cerebral tumor cells seem to use self-tolerance to "trick" immune cells and invade the brain tissue. After years

of research in neuroimmunology, the challenge still remains of gaining a better understanding of how the activity of immune cells is regulated in the CNS, in the hope of finding a safe way to neutralize or stimulate them for therapeutic purposes.

Learning more about how inflammatory responses are induced within the nervous system and the mechanisms by which these responses ultimately contribute to pathology is fundamental in addressing the question of whether inhibition of these responses will be a safe and effective means of reversing or slowing the course of disease. It will be a challenge to design therapeutic agents that safely and effectively target only the detrimental mechanisms that contribute to disease pathogenesis. An understanding of the factors that dictate the switch from a protective to a deleterious inflammatory response will make it possible to devise interventions to limit tissue damage.

This special issue entitled "*Mediators of neuroinflammation,*" features review articles, original research articles, and clinical studies that portray and expand the current knowledge of the specific mediators of neuroinflammation including inducers, sensors, transducers, amplifiers, and effectors of neuroinflammation. The review entitled, "*Cytokines and chemokines at the crossroads of neuroinflammation, neurodegeneration, and neuropathic pain,*" describes how cytokines and chemokines mediate neuroinflammation, with a focus on bacterial meningitis, brain abscesses, Lyme neuroborreliosis, human immunodeficiency virus encephalitis, and neuropathic pain. The protective as well as harmful effects of

cytokines and chemokines are described, with an emphasis on how prolonged inflammation, continual activation and recruitment of effector cells can establish a feedback loop that perpetuates inflammation, and ultimately results in neuronal injury.

There are two review articles that focus on the role of inflammation in ischemia in this special issue. The review entitled, "*TLR2 and TLR4 in the brain injury caused by cerebral ischemia and reperfusion,*" describes the participation of Toll-like receptors, (TLR2 and TLR4) and the resultant downstream signaling pathways that contribute to brain injury caused by cerebral ischemia and reperfusion. The review entitled "*Development and treatments of inflammatory cells and cytokines in spinal cord ischemia-reperfusion injury,*" deals with development of inflammation in spinal cord ischemia-reperfusion injury and reviews the mediators and possible treatment options.

MS, a multifactorial neurological disease characterized by the presence of inflammatory brain infiltrates and subsequent neurodegeneration, is the focus of two review articles in this special issue. The review entitled "*Role of regulatory T cells in pathogenesis and biological therapy of multiple sclerosis,*" outlines the role of regulatory T cells in the pathogenesis and treatment of MS, while the review entitled "*MicroRNAs as novel regulators of neuroinflammation*" describes how microRNAs fine-tune the immune response in MS by functioning as crucial posttranscriptional regulators.

The three research articles featured in this special issue focus on the therapeutic potential of certain drugs in limiting neuroinflammation, hypertension, and demyelination. The dysfunction of the blood-brain barrier (BBB) is a characteristic feature in several CNS disorders including MS. The ability of a quinolizidine alkaloid derivative called Matrine (MAT) in preventing BBB disruption in experimental autoimmune encephalomyelitis, a mouse model of MS, is described in a research article entitled "*Inhibitory effect of matrine on blood-brain barrier disruption for the treatment of experimental autoimmune encephalomyelitis,*" reporting that MAT strengthens BBB integrity by protecting the basement membrane and tight junction proteins by regulating the balance between matrix metalloproteinases (MMP2, MMP9) and tissue inhibitors of metalloproteinases (TIMP1, TIMP2). The therapeutic potential of Scutellarin, a flavone that is used in the Orient as an herbal medication, is evaluated in the research article entitled "*Scutellarin attenuates hypertension-induced expression of brain toll-like receptor 4/nuclear factor kappa B,*" in which a rat model of hypertension is used to describe the role of Scutellarin in lowering blood pressure and promoting neuroprotection by suppressing the proinflammatory TLR4/NF-κB signaling pathway. The aim of the research article entitled "*Sildenafil (Viagra) protective effects on neuroinflammation: the role of iNOS/NO system in an inflammatory demyelination model*" is to study the effect of inducible nitric oxide synthase (iNOS/NO) on inflammatory demyelination and to clarify the neuroprotective effect of Sildenafil (Viagra) using the rat cuprizone model of demyelination, in which oligodendrocyte death and demyelination are independent of immune and inflammatory responses. The authors report that Sildenafil has a direct beneficial effect on oligodendrocytes, protecting these cells and improving myelination. Sildenafil also showed anti-inflammatory effects mainly through iNOS inhibition.

Inflammatory mediators are produced in Alzheimer's disease (AD) and mild cognitive impairment (MCI). Osteopontin (OPN) is a proinflammatory cytokine that has been shown to play an important role in various neuroinflammatory diseases. The clinical study featured in this special issue, entitled "*Elevated osteopontin levels in mild cognitive impairment and alzheimer's disease*", evaluates the correlation between the levels of OPN in the cerebrospinal fluid and plasma and the cognitive deficits in the patients with AD, MCI and other non-inflammatory neurological diseases. The clinical study, entitled "*Eosinophil-derived neurotoxin is elevated in patients with amyotrophic lateral sclerosis*" is aimed at discovering a new diagnostic marker for amyotrophic lateral sclerosis (ALS), a progressive neurodegenerative disease characterized by the loss of certain motor neurons and severe spongy vacuolation of the white matter. Mediators of neuroinflammation, such as members of the family of damage-associated molecular patterns, including reactive oxygen species and eosinophil-derived neurotoxin (EDN), have been shown to play a role in the pathogenesis of ALS. A comparison of the levels of EDN in the serum samples of patients with ALS, AD, and Parkinson's disease and healthy controls revealed a 2.7-fold increase of EDN in ALS patients, suggesting that EDN could serve as a new biomarker for ALS.

Together, the reviews, research articles, and clinical studies that are featured in this special issue enhance our knowledge base of key mechanisms in neuroinflammation.

Acknowledgments

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Research Article

Scutellarin Attenuates Hypertension-Induced Expression of Brain Toll-Like Receptor 4/Nuclear Factor Kappa B

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Hypertension is associated with low-grade inflammation, and Toll-like receptor 4 (TLR4) has been shown to be linked to the development and maintenance of hypertension. This study aimed to investigate the effects of scutellarin (administered by oral gavage daily for 2 weeks) on brain TLR4/nuclear factor kappa B-(NF-κB-) mediated inflammation and blood pressure in renovascular hypertensive (using the 2-kidney, 2-clip method) rats. Immunofluorescence and western immunoblot analyses revealed that hypertension contributed to the activation of TLR4 and NF-κB, accompanied by significantly enhanced expression of proinflammatory mediators, such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-18 (IL-18). Furthermore, expression of the antiapoptotic protein, myeloid cell leukemia-1 (Mcl1), was decreased, and the pro-apoptotic proteins, Bax and cleavedcaspase-3 p17 were increased in combined cerebral cortical/striatal soluble lysates. Scutellarin significantly lowered blood pressure and attenuated the number of activated microglia and macrophages in brains of hypertensive rats. Furthermore, scutellarin significantly reduced the expression of TLR4, NF-κB p65, TNF-α, IL-1β, IL-18, Bax and cleaved-caspase-3 p17, and increased the expression of Mcl1. Overall, these results revealed that scutellarin exhibits anti-inflammatory and anti-apoptotic properties and decreases blood pressure in hypertensive rats. Therefore, scutellarin may be a potential therapeutic agent in hypertension-associated diseases.

1. Introduction

Hypertension is a major risk factor for cardiovascular accidents [1] and may also accelerate the onset and progression of ischemia stroke and cerebral hemorrhage [2]. Hypertension is associated with low-grade inflammation, which may be associated with hypertension-mediated damage to target organs [3]. Inflammation in brain parenchyma can occur as a local process that can be triggered and sustained by activated glial cells, which is thought to contribute to the pathogenesis of several diseases [3]. The innate immune response,

predominantly represented by Toll-like receptors (TLRs), has been shown to contribute to the development of this condition [4].

TLRs are first-line molecules for initiating the innate immune responses, and thus its signaling is involved in the activation of microglia by pathogens and damaged host cells. Activation of microglia by TLRs is considered to be the "classical" form of activation [5]. Activated microglia subsequently secret proinflammatory cytokines and express costimulatory molecules needed for protective immune responses to pathogens and efficient clearance of damaged tissues [6].

TLR4 is an important contributor to microglial activation and known to initiate an inflammatory cascade in response to various brain injuries [7]. TLR4 has been shown to be linked to the development and maintenance of hypertension [8]. Furthermore, TLR4 is involved in cerebrovascular diseases, including stroke [9] and neurodegeneration [10]. TLR4-mediated signaling activates the nuclear factor kappa B (NF-κB) signaling pathway, which plays a critical role in immune and inflammatory responses, cell death, and survival [5, 9]. Therefore, enhanced expression of brain TLR4/NF-κB may play an important role in cerebral pathology induced by hypertension.

5,6,4-Trihydroxyflavone-7-O-glucoronide (scutellarin) is a flavone and the major active component of *Erigeron* breviscapus (Vant.) Hand-Mazz, a herbal medicine in use in the Orient for the treatment of cerebrovascular diseases [11–13]. In recent years, many studies in different animals and cells models have provided evidence for the protective effects of scutellarin because of its antioxidant [12, 14, 15], antiapoptotic [14, 16–18], anti-inflammatory [19, 20], and calcium channel antagonist properties [21]. Therefore, the aim of the present study was to investigate whether scutellarin treatment reduced the expression of brain TLR4/NF-κB, the inflammatory status, and blood pressure in renovascular hypertensive rats.

2. Materials and Methods

2.1. Animals. All experimental procedures were approved by the Institutional Animal Ethical Committee of Sun Yat-Sen University and were conducted according to the Guide for the Care and Use of Laboratory Animal of the National Institute of Health (Publication no. 80-23, revised 1996). A total of 24 male Sprague-Dawley rats (60–80 g) were purchased from the Center for Experimental Animals of Sun Yat-Sen University. Rats were randomly assigned into four groups (six rats per group): (1) sham-operated group (normotensive controls), (2) hypertension with normal saline (NS) treatment, (3) hypertension with low-dose (5 mg/kg per day) scutellarin, and (4) hypertension with high-dose (20 mg/kg per day) scutellarin. Scutellarin Scutellarin (Yunnan Biovalley pharmaceutical Company Ltd, Yunnan, China) was dissolved in sterile NS, and different doses of scutellarin were administered by gavage lasting for 2 weeks.

2.2. Hypertension Model and Drug Administration. Hypertension was induced using a 2-kidney, 2-clip method (2K2C), as described by Zeng et al. [22]. Briefly, under anesthesia with 10% chloral hydrate (3 mL/kg body weight, intraperitoneally [i.p.]), a median longitudinal incision on the abdominal skin was performed, and then the roots of both right and left renal arteries were constricted by placing ring-shaped silver clips with an inner diameter of 0.30 mm to induce hypertension. Approximately 8 weeks later, those rats with systolic blood pressure higher than 140 mmHg and without stroke symptoms were selected for the experiment. Different doses of scutellarin were administered by gavage daily for 2 weeks. In the NS group, hypertensive rats were given saline

in the same volume as scutellarin. Renal arteries in sham-operated rats also underwent surgery but without placement of clips. Systolic blood pressure (SBP) was measured by an indirect tail-cuff sphygmomanometer (MRB-IIIA, Shanghai Institute of Hypertension, Shanghai, China) in conscious rats heated (heat lamp at 37°C, for 5 min) before and after renal artery constriction (at weekly intervals) for 10 weeks [23].

2.3. Preparation of Tissue Samples. The preparation of tissue samples was performed as described previously [24, 25]. Briefly, after 2 weeks of scutellarin treatment, rats were sacrificed under deep anesthesia with 10% chloral hydrate (5 mL/kg body weight, i.p.) and then transcardially perfused with 0.9% sodium chloride (at 4°C). The brains were removed, and the left frontal cerebral cortex and striatum were rapidly dissected and used for western immunoblotting analysis. For immunofluorescence labeling, the right frontal brain was sliced into horizontal sections (10 μm thick) using the CM1900 cryostat (Leica, Heidelberg, Germany), and these section, were then fixed with 4% paraformaldehyde (in 0.01 M phosphate-buffered saline (PBS), pH 7.4).

2.4. Immunofluorescence Labeling. Immunofluorescence was carried out as described previously [7]. Briefly, sections were preincubated with 0.3% Triton X-100 in 0.01 M PBS (10 min), blocked with 10% normal goat serum (KPL, CA, USA) (1 h at room temperature RT), and then incubated (overnight at 4°C) with the primary antibodies (in primary antibody diluents (Dako, Denmark)): rabbit anti-TLR4 (1 : 100) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse anti-Neuronal Nuclei (NeuN) (1 : 400) (Chemicon, USA), mouse anti-rat glial fibrillary acid protein (GFAP) (1 : 800) (Cell Signaling Technology, Beverly, MA, USA), or mouse anticluster of differentiation 11b (CD11b or OX-42) (1 : 300) (Millipore, USA). Sections were then incubated with Alexa Fluor 555 conjugated goat anti-rabbit IgG (H + L), F(ab$'$)2 Fragment (1 : 1000), Alexa Fluor 555 conjugated goat anti-mouse IgG (H + L), F(ab$'$)2 Fragment (1 : 1000) (both from Cell Signaling Technology), or fluorescein isothiocyanate-goat anti-mouse IgG antibodies (1 : 200) (Zymed, USA) in 0.01 M PBS (1 h at RT). Sections counterstained for nuclei were exposed to 4$'$,6-diamidino-2-phenylindole dihydrochloride (1 : 1000) (Roche, Mannheim, Germany) and then mounted in ProLong Gold antifade reagent (P36930, Invitrogen, USA) prior to imaging. Immunoreactivity was visualized using the BX51 microscope (Olympus). Negative control sections were incubated with PBS only, and showed no positive staining (data not shown).

2.5. Western Immunoblot Analyses. Western immunoblotting was performed as previously described [26]. Briefly, the left frontal cerebral cortex and striatum were homogenized in lysis buffer (pH 7.6) with 0.01 μg/mL phenylmethanesulfonyl fluoride centrifuged (16,400 rpm for 30 min), and then Protein concentrations were determined using a bicinchoninic acid (BCA) protein assay kit (PIERCE, USA) according to the manufacturer's instructions. Soluble protein (50 μg) was separated by 4–20% gradient SDS/PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) (Bio-Rad) and then transferred onto polyvinylidene fluoride membranes

(Millipore, USA). Membranes were blocked with 5% skim milk in Tris-buffered saline containing 0.1% Tween-20 and then incubated with the primary antibodies: rabbit anti-TLR4 (1 : 1000), rat anti-interleukin 1 beta (IL-1β) (1 : 1000), rabbit anti-myeloid cell leukemia-1 (mcl1) (1 : 1000) (all from Abcam, USA), mouse anti-NF-κB p65 (1 : 1000) (Cell Signaling Technology, USA), goat anti-tumor necrosis factor alpha (TNF-α) (1 : 5000) (Novus Biologicals, USA), rabbit anti-IL-18 (1 : 300), rabbit anti-Bax (1 : 300), or mouse anti-caspase-3 p17 (1 : 300) (all from Santa Cruz Biotechnology, USA). Membranes were exposed to the secondary antibodies diluted in blocking buffer for 1 h at room temperature: horseradish peroxidase (HRP)-conjugated goat anti-mouse (1 : 6000) (EarthOx, USA), HRP-conjugated goat anti-rabbit (1 : 3000) (Cell Signaling Technology), or HRP-conjugated rabbit anti-goat IgG antibodies (1 : 3000) (Invitrogen, USA). Mouse monoclonal anti-β-actin (1 : 3000) (Proteintech Group Inc., USA) served as the housekeeping protein. Immunoreactive bands were detected using Chemiluminescent HRP Substrate (Millipore, USA) and visualized on Kodak X-OMAT films. The optical densities were normalized to those of β-actin and calculated as target protein expression/β-actin expression ratios (using Image J 1.42q).

2.6. Image Analysis and Quantification. All histological images were analyzed with Image-Pro Plus image analysis software (Media Cybernetics, Silver Spring, MD, USA) by one blinded assessor. The number of positively stained cells was counted using Image-Pro Plus image analysis software in nine comparable, nonoverlapping fields (425 μm × 320 μm; 3 fields per section × 3 sections per rat) and was presented as the average cell number per field on each section [27, 28].

2.7. Statistical Analysis. All data are expressed as the mean ± standard deviation and were analyzed by one-way analysis of variance (ANVOA) followed by the least significant difference (LSD) post hoc test. Significance was reached at values of $P < 0.05$ and $P < 0.001$. Statistical analysis was performed with Statistical Product and Service Solutions (SPSS) 13.0 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Effect of Scutellarin Treatment on SBP. Baseline SBP was similar between the four groups. SBP was only slightly increased in hypertension-induced rats but increased progressively to 174.7 ± 13.9, 180.9 ± 6.2, 178.8 ± 6.7, and 126.4 ± 9.8 mmHg in NS, low-dose, high-dose, and sham-operated groups, respectively (Figure 1). SBP in the NS, low-dose, and high-dose groups was significantly higher compared with the sham-operated group ($P < 0.001$). There were no incidences of stroke or death in the four groups (Table 1). No significant difference in SBP was found before treatment in NS, low-dose and high-dose groups. Compared with the NS group (196.5 ± 9.8 mmHg), scutellarin treatment significantly reduced SBP in a dose-dependent manner ($P < 0.001$). In the low-dose group, SBP was decreased by approximately 11.5 ± 6.5 mmHg, from 180.9 ± 6.2 mmHg to 169.1 ± 7.1 mmHg. In the high-dose group, SBP was reduced by approximately 17.2 ± 7.4 mmHg,

Group	Baseline	Before treatment	After treatment
Sham	102.7 ± 7.8	126.4 ± 9.8	137.2 ± 8.3
NS	99.4 ± 7.0	174.7 ± 13.9	196.5 ± 9.8
Low dose	100.2 ± 6.8	180.9 ± 6.2	169.1 ± 7.1
High dose	101.4 ± 5.9	178.8 ± 6.7	161.2 ± 9.9

FIGURE 1: Effect of scutellarin treatment on systolic blood pressure (SBP). Baseline systolic blood pressure (SBP) (mmHg) was similar among the four experimental groups. SBP was significantly increased in hypertensive groups compared with the sham-operated group. No significant difference in SBP was found in NS rats prior to treatment. However, SBP was significantly reduced with low- and high-dose scutellarin. $^*P < 0.001$ versus sham group; $^\#P > 0.05$, $^\blacktriangle P < 0.001$ versus NS group; $^\blacklozenge P < 0.001$, versus before treatment; $^\blacktriangledown P > 0.05$, $^\& P < 0.001$ versus low-dose group.

TABLE 1: Occurrence of stroke and death in different groups of rats. There were no incidences of stroke or death in the four groups.

Group	Stroke				Death
	CH	CI	CH + CI	SAH	
Sham	0	0	0	0	0
NS	0	0	0	0	0
Low dose	0	0	0	0	0
High dose	0	0	0	0	0

CH: cerebral hemorrhage; CI: cerebral infarction; SAH: subarachnoid hemorrhage.

from 178.8 ± 6.7 mmHg to 161.2 ± 9.9 mmHg. Furthermore, SBP was significantly different between the low-dose and high-doses group ($P < 0.001$) and was significantly higher compared with the sham-operated group (137.2 ± 8.3 mmHg) ($P < 0.001$).

3.2. Scutellarin Decreased the Number of Activated Microglia/ Macrophages in Hypertensive Rats. OX-42 labels both microglia and macrophages. Chronic hypertension induced

(a)

(b)

(c)

(d)

(e)

FIGURE 2: Scutellarin decreases the number of activated microglia in brain of hypertension. (a–d) Cluster of differentiation 11b (OX-42) immunostaining for microglia. (e) Quantification of OX-42-positive cells revealed that activated microglia were significantly increased in normal saline (NS) rats compared with the sham group, and treatment with scutellarin significantly reduced these numbers in a dose-dependent manner. $^*P < 0.001$ versus sham group; $^\blacklozenge P < 0.001$ versus NS group; $^\& P < 0.001$ versus low-dose group. Scale bar = 100 μm.

microglia/macrophage activation (Figure 2). Compared with the sham group, the number of cells positively stained with OX-42 was significantly increased in the NS group ($178.7 \pm 18.5/mm^2$ versus $86.2 \pm 16.8/mm^2$) ($P < 0.001$). In contrast, the number of cells positively stained with OX-42 was significantly decreased with low-dose and high-dose scutellarin, $143.1 \pm 21.9/mm^2$ and $117.4 \pm 17.8/mm^2$, respectively ($P < 0.001$). Furthermore, counts in the high-dose group were significantly lower compared with the low group ($P < 0.001$).

3.3. Scutellarin Suppressed Hypertension-Induced Expression of Brain TLR4.
TLR4 immunoreactivity was sparse in the cerebral cortex and striatum in sham-operated rats (Figure 3(a); sham). In contrast, chronic hypertension induced higher TLR4 expression in these regions (Figure 3(a); hypertension). TLR4 was further investigated for its cellular distribution using markers for neurons (NeuN), astrocytes (GFAP) and microglia (OX-42). The majority of TLR4 (red) was colabeled with OX-42-positive cells (green) (Figure 3(b); (A)). In contrast, few TLR4-positive cells were co-labeled with GFAP-positive (green) (Figure 3(b); (B)) or NeuN-positive (green) (Figure 3(b); (C)) cells. Western immunoblot analysis showed that compared with the sham-operated group, levels of TLR4 protein were significantly increased (approximately 6-fold) in the
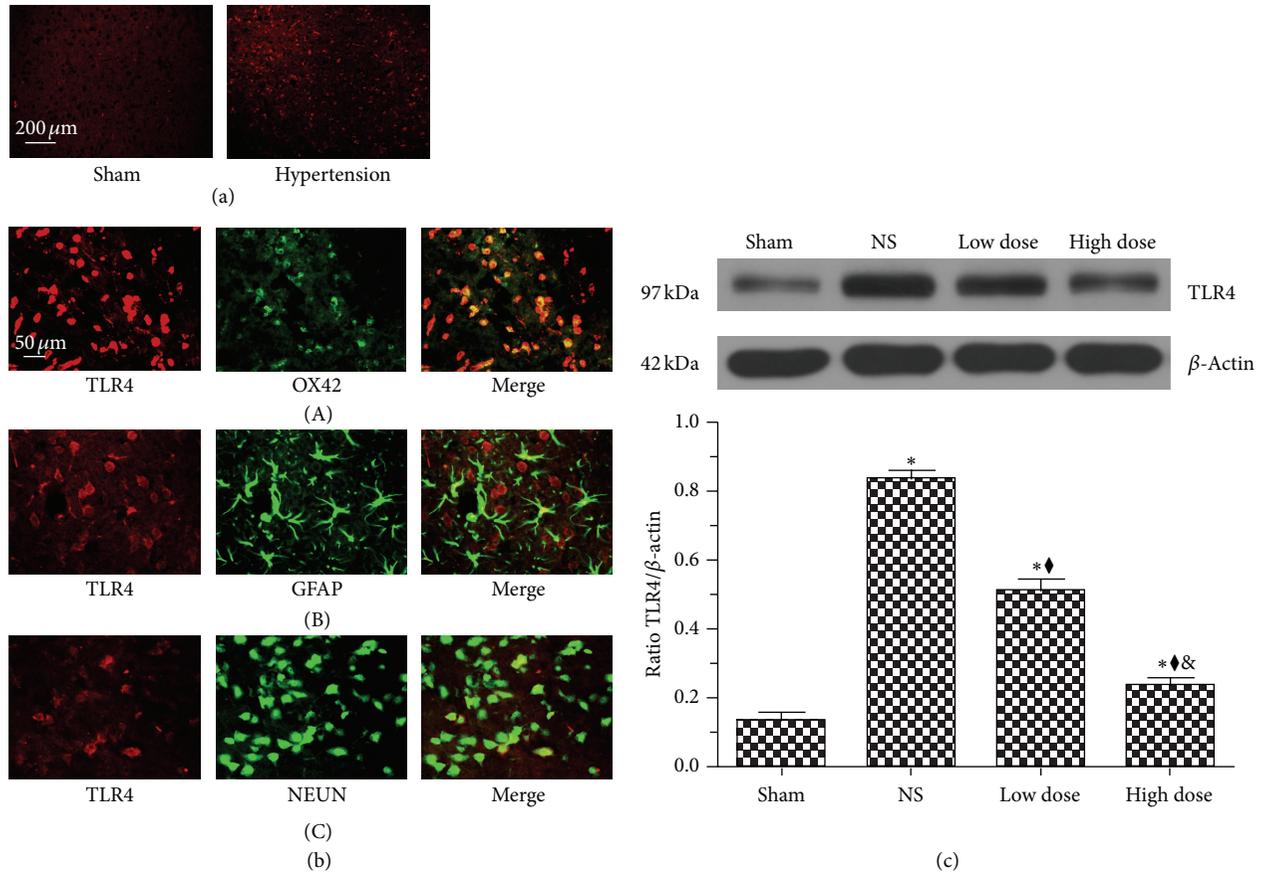
FIGURE 3: Scutellarin suppresses hypertension-induced expression of brain Toll-like receptor 4 (TLR4). (a) Chronic hypertension significantly enhanced the expression of TLR4 in the cerebral cortex. (b) Double immunofluorescence labeling indicated that the majority of TLR4 (red) was colabeled with OX-42-positive (green) cells and fewer with glial fibrillary acid protein- or Neuronal Nuclei-positive cells. (c) Western immunoblot analysis of brains of hypertensive rats showed that scutellarin dose-dependently suppressed the expression of TLR4. $^*P < 0.001$ versus sham group; $^{\blacklozenge}P < 0.001$ versus NS group; $^{\&}P < 0.001$ versus low-dose group. (c) Scale bar = 200 μm (a) and 50 μm (b).

NS group (Figure 3(c)) ($P < 0.001$). However, treatment with scutellarin significantly decreased this level in a dose-dependent manner, approximately 39.9% and 72.1% in the low-dose and high-dose groups, respectively (Figure 3(c)) ($P < 0.001$).

3.4. Scutellarin Attenuated Hypertension-Induced Expression of NF-κB, TNF-α, IL-1β, and IL-18.

TLR4 mediates the activation of transcription factors, such as NF-κB, which subsequently induces the production of inflammatory cytokines. In the present study, western immunoblot analysis showed that compared with the sham group, protein levels of NF-κB p65, TNF-α, IL-1β, and IL-18 in NS rats were significantly increased by approximately 7-, 6.5-, 3.7-, and 4-fold, respectively (Figures 4(a)–4(d)) ($P < 0.001$). These proteins were significantly reduced by scutellarin, with the high-dose group inducing a markedly higher attenuation compared with the low-dose group (Figures 4(a)–4(d)) ($P < 0.001$). Levels of NF-κB p65, TNF-α, IL-1β, and IL-18 were reduced to 51.1%, 61.2%, 82.9%, and 83.3%, respectively, in the low-dose group, in contrast to 31.4%, 41.9%, 57.8%, and 53.4%, respectively, in the high-dose group. Therefore, these results suggest that

scutellarin reduced hypertension-mediated induction of the inflammatory response.

3.5. Scutellarin Treatment Upregulated the Expression of Mcl1, and Suppressed Bax and Caspase-3 p17.

To investigate the potential effect of scutellarin on neuronal cell survival, we evaluated the expression of the apoptosis-related proteins, Mcl1, Bax, and cleavedcaspase-3 p17 in brains of hypertensive and sham-operated rats. Western immunoblot analysis indicated that compared with the sham group, levels of Mcl1, Bax, and cleavedcaspase-3 p17 were significantly increased in hypertensive rats (Figures 5(a)–5(c)) ($P < 0.001$). Compared with sham rats, Mcl1, Bax, and cleavedcaspase-3 p17 were significantly ($P < 0.001$) elevated in the NS group by approximately 2-, 3.8-, and 8.9-fold, respectively. Compared with the NS group, treatment with scutellarin significantly upregulated the level of Mcl1, particularly with the high-dose group (approximately 2.1-fold compared with the NS group) (Figure 5(a)) ($P < 0.001$). However, scutellarin significantly downregulated the levels of Bax and cleavedcaspase-3 p17 protein in a dose-dependent manner, to 71.4% and 73.9% (for Bax and cleavedcaspase-3 p17, resp.) for the low-dose group

(a)

(b)

(c)

(d)
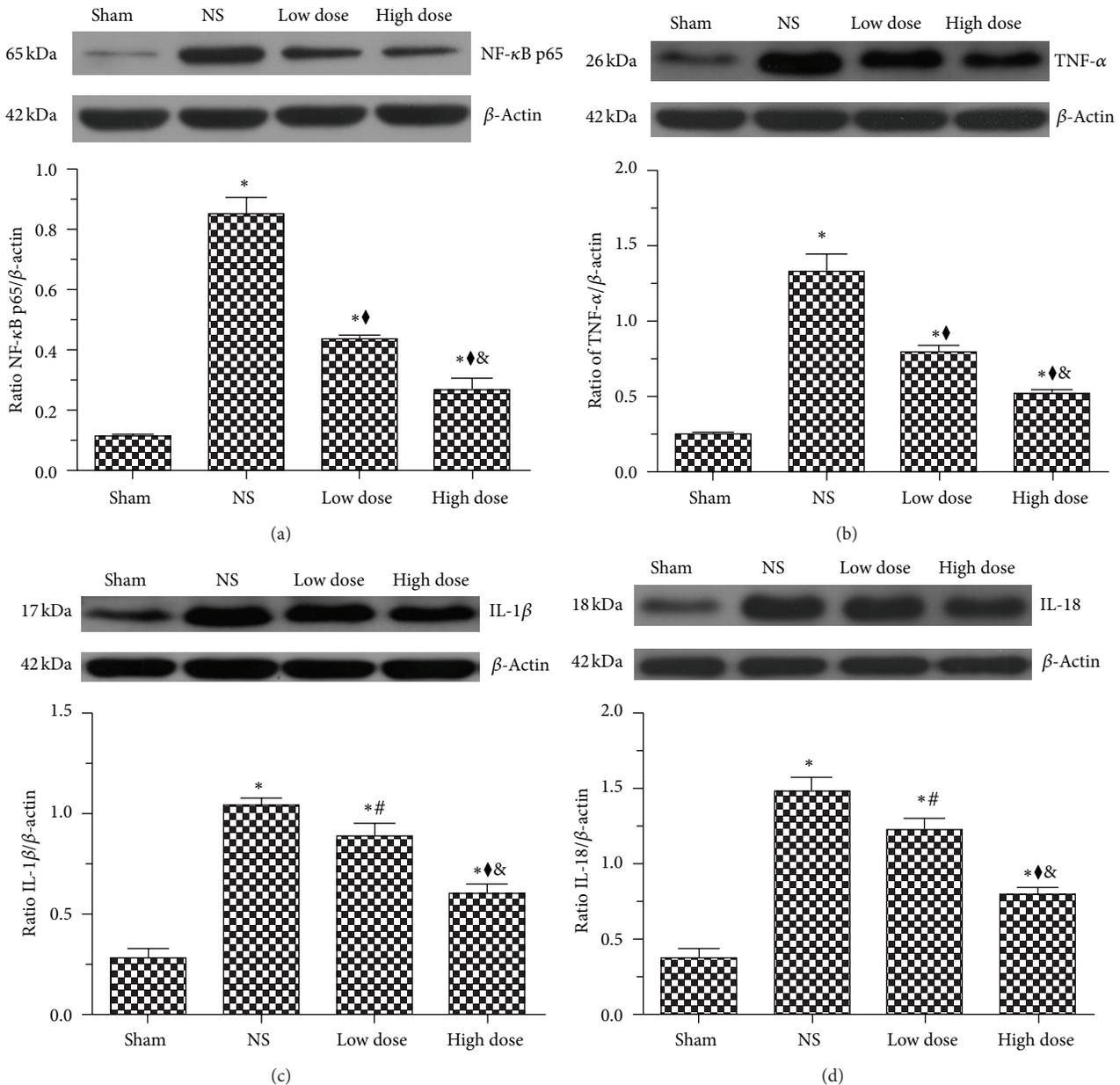
FIGURE 4: Scutellarin attenuates hypertension-induced brain expression of NF-κB, TNF-α, IL-1β, and IL-18. Western immunoblot analysis for (a) NF-κB p65, (b) TNF-α, (c) IL-1β, and (d) IL-18 in the rat cortex and striatum. Treatment with scutellarin significantly reduced the expression of these inflammatory markers in a dose-dependent manner. $^{*}P < 0.001$ versus sham group; $^{#}P < 0.05$, $^{\blacklozenge}P < 0.001$ versus NS group; $^{\&}P < 0.001$ versus low-dose group.

and 56.3% and 27.1% (for Bax and cleavedcaspase-3 p17, resp.) for the high-dose group (Figures 5(a) and 5(c)) ($P < 0.001$).

4. Discussion

In the present study, we demonstrate that scutellarin is protective against chronic hypertension-induced activation of brain TLR4 and subsequent NF-κB-mediated inflammatory responses. We show that scutellarin possesses anti-inflammatory and antiapoptotic properties and lowers blood pressure, thus suggesting its use as a potential therapeutic agent in hypertension-associated diseases.

In the central nervous system, TLR4 has been reported to be expressed in both microglia and astrocytes, as well as in neurons [7]. In this study, chronic hypertension augmented the expression of TLR4 predominantly in microglia/macrophage cells, indicating its involvement in chronic hypertension-induced inflammation. However, the expression of TLR4 in astrocytes and neurons also suggested their potential involvement, and thus further studies could explore this possible relationship.

Innate and adaptive immunities have been shown to contribute to hypertension-associated end-organ damage, although the mechanism by which this occurs remains

(a)

(b)

(c)
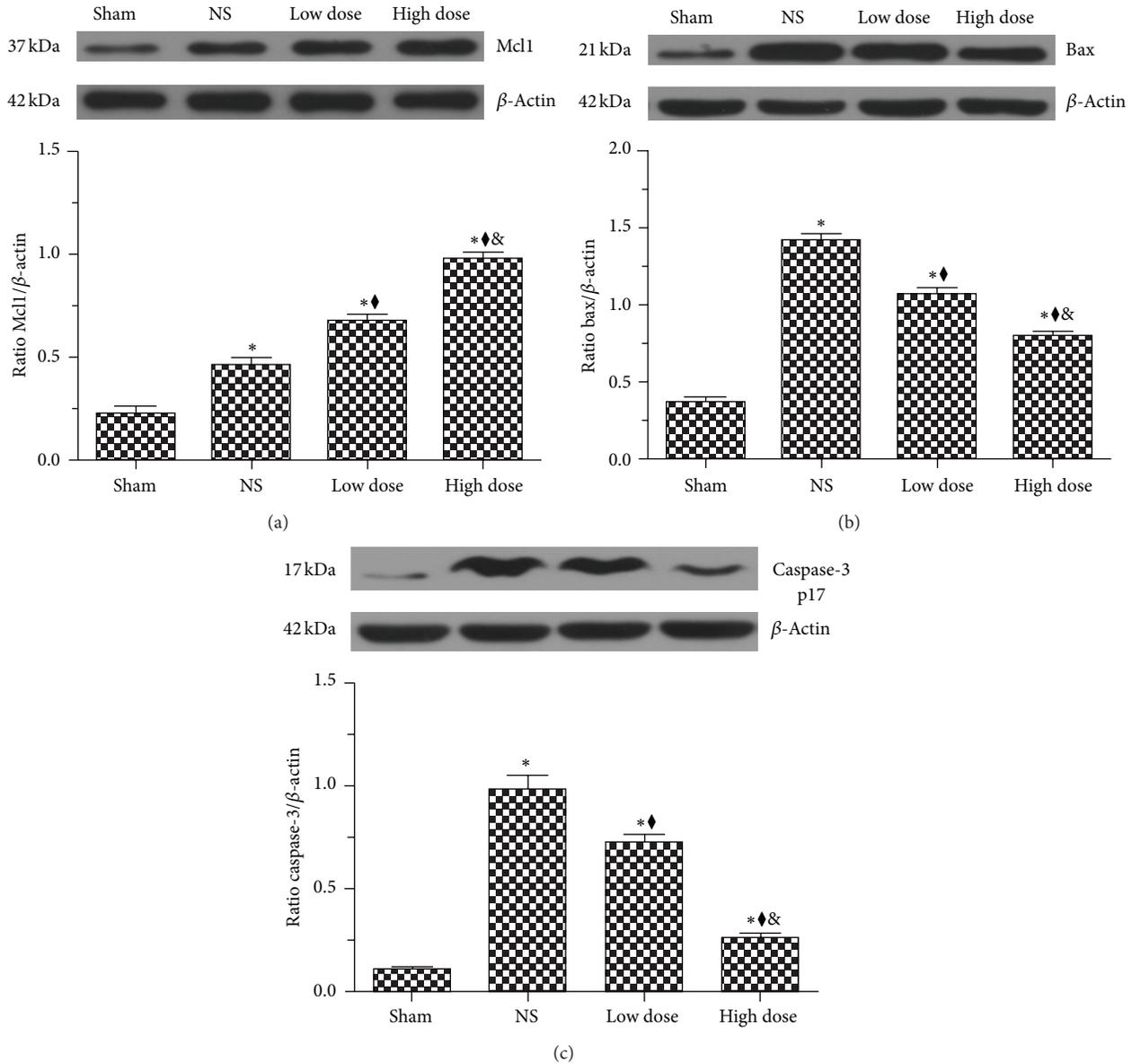
Figure 5: Scutellarin treatment upregulated Mcl1 and suppressed Bax, caspase-3 p17 expression. Western immunoblot analysis of protein levels of (a) Mcl1, (b) Bax, and (c) cleavedcaspase-3 p17 in the rat cortex and striatum. Scutellarin significantly upregulated Mcl1 but dose-dependently decreased Bax and cleavedcaspase-3 p17 (c). $^{*}P < 0.001$ versus sham group; $^{\blacklozenge}P < 0.001$ versus NS group; $^{\&}P < 0.001$ versus low-dose group.

unclear [29]. Previous studies suggest that enhanced expression of TLR4 may be linked with the development and maintenance of hypertension and low-grade inflammation and augmented vascular contractility in hypertensive rats [8, 29]. Chronic hypertension causes cardiac hypertrophy, characterized by low-grade inflammation and accompanied by increased expression and activity of TLR4, and elevated gene expression of TNF-α and IL-6 in cardiac tissue [8]. Treatment with anti-TLR4 was shown to decrease mean arterial pressure, TLR4 protein in mesenteric resistance arteries, and serum levels of IL-6 in spontaneously hypertensive rats [29]. Furthermore, TLR4 signaling is also involved in brain damage and in neuroinflammatory processes associated with

ischemic stroke and neurodegenerative diseases, such as Alzheimer's disease [10, 30–32]. Neutralizing TLR4 at the time of intracerebral hemorrhage [7] and ischemic stroke [31] provides neuroprotection. This effect may result from TLR4-mediated activation of NF-κB signaling pathways linked to the transcription of many proinflammatory genes encoding for cytokines, chemokines, proteins of the complement system, and cell adhesion molecules. Findings from our study of chronically hypertensive rats revealed that in addition to reducing blood pressure, scutellarin prevented inflammatory mediated neuronal damage by suppressing microglial activation and the concomitant rise in expression of NF-κB, TNF-α, IL-1β, and IL-18. The underlying mechanism involves, at

least in part, inhibition of TLR4/NF-κB-dependent signaling pathway. Interestingly, although treatment with scutellarin decreased SBP, the antihypertensive effect was moderate and without a dose-response relationship, suggesting that the low dose of scutellarin may have already reached the maximum antihypertensive effect. Thus, the antihypertensive action may play a minor role in the protective activity of scutellarin against hypertension-induced brain inflammation. Therefore, these results suggest that TLR4 is a promising target for the prevention and treatment of hypertension-associated diseases.

Studies in rat primary microglia and BV2 mouse microglia cell lines have shown that scutellarin inhibits LPS-induced nuclear translocation and DNA binding activity of NF-κB, accompanied by reduced production of proinflammatory mediators, such as TNF-α and IL-1β [11]. Furthermore, recent reports have demonstrated the protective effects of scutellarin in the brain and heart of ischemic rats [16, 33]. In line with these results, the present study found that scutellarin decreased hypertension-mediated neuronal apoptosis, possibly resulting from reduced TLR4- and NF-κB-mediated production of the proinflammatory cytokines.

Scutellarin is a small molecule, and its neuroprotective effects have been well documented in different brain disease models [13, 33]. The present study further demonstrated its anti-inflammatory and antiapoptotic action in the hypertensive brain. However, the precise molecular mechanism by which scutellarin protects against hypertension-induced brain damage still remains elusive. Further study on the protective molecular mechanisms, pharmacokinetics and brain penetration of scutellarin will help explain its limited effects on blood pressure and provide relevant evidence for future clinical applications.

5. Conclusions

In summary, chronic hypertension significantly enhanced the expression of TLR4, NF-κB, and the production of the proinflammatory cytokines, TNF-α, IL-1β, and IL-18 in brains of hypertensive rats. Scutellarin lowered blood pressure and provided neuroprotective effects by suppressing TLR4/NF-κB-mediated inflammation. Therefore, scutellarin may have therapeutic potential against hypertension-associated diseases.

Conflict of Interests

The authors have no conflict of interests to disclose.

Authors' Contributions

Xingyong Chen and Xiaogeng Shi contributed to in vivo experiments, RHR model, Scutellarin administration, immunofluorescent labeling, tissue preparation, western blot analyses, statistical analyses, and first draft of paper. Simei Long contributed to western blot analyses. Xiaogeng Shi and Zhong Pei contributed to design of study, conception of study, revision of paper, and final approval of paper. Ruxun Huang: design of study, financial support, revision of paper,

final approval of paper. Xu Zhang, Huixin Lei, and Huanxing Su contributed to revision of paper. All authors read and approved the final paper. Xingyong Chen and Xiaogeng Shi contributed equally to this work.

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Research Article

Inhibitory Effect of Matrine on Blood-Brain Barrier Disruption for the Treatment of Experimental Autoimmune Encephalomyelitis

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Dysfunction of the blood-brain barrier (BBB) is a primary characteristic of experimental autoimmune encephalomyelitis (EAE), an experimental model of multiple sclerosis (MS). Matrine (MAT), a quinolizidine alkaloid derived from the herb Radix Sophorae Flave, has been recently found to suppress clinical EAE and CNS inflammation. However, whether this effect of MAT is through protecting the integrity and function of the BBB is not known. In the present study, we show that MAT treatment had a therapeutic effect comparable to dexamethasone (DEX) in EAE rats, with reduced Evans Blue extravasation, increased expression of collagen IV, the major component of the basement membrane, and the structure of tight junction (TJ) adaptor protein Zonula occludens-1 (ZO-1). Furthermore, MAT treatment attenuated expression of matrix metalloproteinase-9 and -2 (MMP-9/-2), while it increased the expression of tissue inhibitors of metalloproteinase-1 and -2 (TIMP-1/-2). Our findings demonstrate that MAT reduces BBB leakage by strengthening basement membrane, inhibiting activities of MMP-2 and -9, and upregulating their inhibitors. Taken together, our results identify a novel mechanism underlying the effect of MAT, a natural compound that could be a novel therapy for MS.

1. Introduction

Multiple sclerosis (MS) and its animal model, experimental autoimmune encephalomyelitis (EAE), are T cell-mediated inflammatory diseases characterized by lymphocyte infiltration, demyelination, and axonal injury [1, 2]. Although MS pathology is not fully understood, blood-brain barrier (BBB) dysfunction plays an essential role in the pathogenesis of this disease. In both MS and EAE, proinflammatory cells and toxic molecules migrate into the brain via the damaged BBB, resulting in cerebral edema, demyelination, and neural cell death [3, 4].

The BBB is composed of basement membrane, interendothelial tight junctions (TJs), and perivascular astrocytes [5]. The basement membrane, which is composed of two distinct types, namely, endothelial basement membrane and parenchymal basement membrane, is a tight assembly of specialized extracellular matrix molecules [6]. This membrane, together with the endothelial cell monolayer, forms a structural barrier that selectively filters blood elements [6, 7]. Collagen IV comprises 90% of total protein in the basement membrane and plays a decisive role in maintaining the structural integrity of the vessel wall [8, 9]. Collagen IV, as a major component of the cerebral microvascular basal lamina, is widely used as a marker to determine the extent of destruction of the basement membrane.

TJs, composed of large multiprotein complexes, seal the gaps between biological barriers [4]. Altered distribution or loss of TJs is frequently seen in ischemic cerebral microvessels, resulting in diminished BBB integrity [10]. Zonula occludens-1 (ZO-1) is the primary cytoplasmatic protein associated with TJs, which links the C-terminal ends of occludin and claudins to the underlying actin cytoskeleton [7]. A decrease in ZO-1 expression results in increased BBB

permeability [11]. In addition, disease severity during the acute phase of EAE is directly associated with the extent of BBB permeability [12].

It has been shown that BBB disruption is accompanied by excessive expression of matrix metalloproteinases (MMPs) [13]. MMPs, including MMP-9 and MMP-2, belong to a class of zinc-bound proteases, whose functions include induction of inflammation, cleavage of myelin proteins, activation or degradation of disease-modifying cytokines, and direct damage to CNS cells [14]. Abnormal increases in MMP-9 and MMP-2 in endothelial cells may collectively impair endothelial barrier function by degrading the vascular basement membrane and TJs [10, 14, 15]. Furthermore, MMP-9 and MMP-2 are upregulated in the CNS of rat models of EAE [16].

Tissue inhibitors of metalloproteinases (TIMPs) are endogenous inhibitors of MMPs. TIMP-1 controls MMP-9 activity through high affinity, noncovalent binding to the MMP catalytic domain, whereas MMP-2 is bound by TIMP-2 [17]. It has been shown that TIMP-1 deficiency enhances disease severity during EAE [18]. Under normal physiological conditions, there is a constant balance between MMP and TIMP activity, which is essential in maintaining the physiological functions of the organism [19]. In contrast, an imbalance in MMP/TIMP ratio is found in various pathological conditions in humans, such as cancer, rheumatoid arthritis, and vascular diseases [20]. For example, the serum MMP-9/TIMP-1 ratio in relapsing-remitting MS patients correlates with development of the disease [17]. An imbalance between MMP-2 and TIMP-2 caused by radiation plays a role in the pathogenesis of brain injury [21].

Currently, treatment of MS is limited to immunomodulatory or immunosuppressive therapy, which is not always successful and often has severe side effects [22]. Hence, the search for more effective and more tolerable compounds is of great importance. Matrine (MAT) is a natural alkaloid component extracted from the herb Radix Sophorae Flaves, with a MW of 258.43 ($C_{15}H_{24}N_2O$, Figure 1(a)). It has been reported that MAT suppressed immune activities of T cells, B cells, and macrophages [23]. Matrine has long been used for the treatment of viral hepatitis, cardiac arrhythmia, and skin inflammation, without known side effects [24, 25]. While MAT suppressed development of EAE, its mechanism for neuroprotection has not been elucidated. The purpose of this study was to determine whether MAT treatment inhibits BBB disruption by reducing BBB leakage, strengthening the basement membrane, enhancing TJs, and regulating the balance between MMPs and TIMPs during disease progression of EAE.

2. Materials and Methods

2.1. Animals. Female, 6-7 week-old Wistar rats were purchased from Shanghai Xipuer-Bikai Experimental Animal Company, China, and housed in the aseptic laboratory of the Experimental Animal Center of Henan, China. All efforts were made to minimize the numbers of animals used and to ensure minimal suffering.

2.2. EAE Induction and Treatment. EAE was induced as described previously [23] with only minor modifications. Spinal cord homogenate of guinea pig (Experimental Animal Center of Hebei) weighing 300–350 g was emulsified with the same volume of complete Freund's adjuvant (CFA) (Sigma, USA) containing 6 mg/mL Bacillus Calmette-Guérin vaccine (Shanghai Institute of Biological Products, China). Each rat received a subcutaneous injection of 0.5 mL emulsion divided among 5 sites draining into the nape and back. All procedures were approved by the Bioethics Committee of Zhengzhou University.

Immunized rats were randomly divided into four groups ($n = 16$ each group) for different treatments. Briefly, MAT (Jiangsu Chia Tai Tianqing Pharmaceutical Co., Jiangsu, China) was dissolved in normal saline and injected intraperitoneally (i.p.) daily at two doses: low (150 mg/kg; MAT-L) and high (250 mg/kg; MAT-H), the dosage calculated at 6.7 mL/kg, from day 1 until day 17 after immunization (p.i.). Dexamethasone (DEX) (Henan Hongrun Pharmaceutical Co., Henan, China), as the positive control drug, was dissolved in normal saline (6.7 mL/kg) and injected (i.p.) daily, from day 1 until day 17 p.i. at 1 mg/kg. Immunized rats that received the same amount of normal saline only i.p. served as a vehicle control, and 16 nonimmunized naive rats that received the same amount of normal saline i.p. served as the naive group.

2.3. Clinical Scoring and Weight. Rats were monitored and weighed daily by two independent observers to evaluate clinical scores of EAE after immunization. Neurological signs were assessed as follows [23]: 0 = no clinical score; 1 = loss of tail tone; 2 = hind limb weakness; 3 = hind limb paralysis; 4 = forelimb paralysis; 5 = moribund or death.

2.4. Histopathological Evaluation. On day 17 p.i., two independent observers randomly selected 8 rats from each group. When animals were sacrificed, sera and spinal cords were collected after extensive perfusion. The lumbar enlargement of spinal cords was embedded in paraffin. After embedding, 2-3 μm thick sections were prepared and stained with hematoxylin-eosin (HE) for inflammatory infiltration and chromotrope 2R-brilliant green (C-2R-B) for demyelination. Histopathological examination was performed and scored in a blinded fashion as follows [22]: for inflammation: 0, no inflammatory cells; 1, a few scattered inflammatory cells; 2, organization of inflammatory infiltrates around blood vessels; 3, extensive perivascular cuffing with extension into adjacent parenchyma, or parenchyma; for demyelination: 0, none; 1, rare foci; 2, a few areas of demyelination; 3, large (confluent) areas of demyelination.

2.5. Evaluation of BBB Leakage. BBB leakage was assessed using Evans Blue (EB) dye as previously described [26]. On day 17 p.i., the 8 rats remaining in each group were anesthetized. EB dye (2%; 4 mL/kg; Sigma, St. Louis, MO, USA) was injected slowly into the tail vein and was allowed to circulate for 60 minutes. When the rats were sacrificed, brains were removed and immediately weighed. The EB
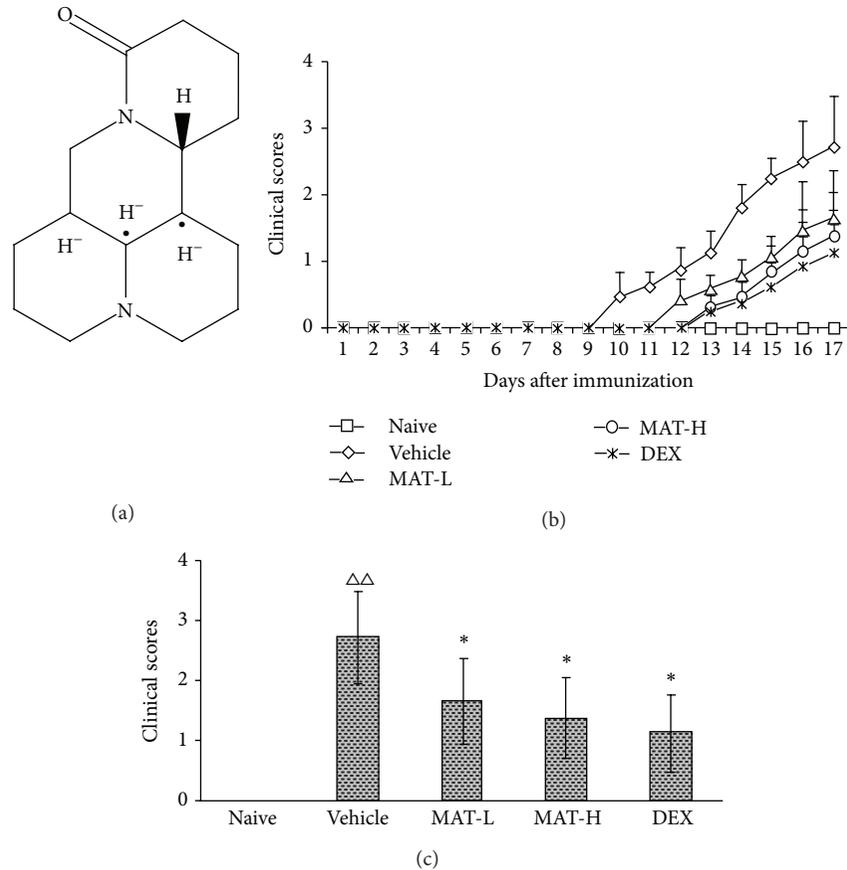
FIGURE 1: MAT reduces clinical signs of EAE. (a) Structure of MAT. (b) Effect-versus-time curves of MAT effects on EAE. Clinical EAE was scored daily after immunization according to a 0–5 scale. (c) The mean maximum clinical scores are expressed as mean ± SD ($n = 16$ each group). $^{\triangle\triangle}P < 0.01$, compared to naive group; $^{*}P < 0.05$, compared to vehicle-treated group.

dye was extracted in 2.5 mL of PBS, and 2.5 mL of 60% trichloroacetic acid was added. The mixture was then vortexed and centrifuged for 40 min at 4000 rpm, and the amount of EB dye in supernatants was determined at 610 nm by spectrophotometry and quantified to μg/g brain tissue.

2.6. RT-PCR Analysis of Collagen IV and ZO-1 mRNAs. The cervical spinal cords were harvested on day 17 p.i. and were prepared for analysis of collagen IV and ZO-1 mRNA using real-time polymerase chain reaction (PCR). Total cellular RNA from these tissues was isolated using TRIzol reagent (Beijing TransGen Biotech Co., Beijing, China) following the standard protocol. cDNA synthesis was performed by reverse transcription using a Promega reverse transcription kit. The cDNA copy number for each gene was determined using standard curves of the corresponding PCR product. Primers for collagen IV were 5′-GGCCCCTGCTGAAG-CGTT-3′ (forward) and 5′-GTTCCCCGAGCACCTTAG-3′ (reverse), which produced a 306 bp PCR product. Primers for ZO-1 were 5′-CCATCTTTGGACCGATTGCTG-3′ (forward) and 5′-TAATGCCCGAGCTCCGATG-3′ (reverse), which produced a 372 bp PCR product. Gene expression was normalized to expression of the endogenous housekeeping gene β-actin. The β-actin primers were 5′-CCTCTGAACCCTAAGGCCAAC-3′ (forward) and 5′-TGCCACAGGATTCCATACC-3′ (reverse), which produced

a 564 bp PCR product. To determine the relative quantification of target gene expression, we used the gel imaging analysis system (Dalian Jingmai Biotech Co., Liaoning, China).

2.7. ELISA Analysis of MMP-9 and TIMP-1. Serum collected on day 17 p.i. was assayed for concentrations of MMP-9 and TIMP-1 by ELISA following the manufacturer's instructions (R & D Systems, USA). Samples were quantified by comparison with the standard curves of MMP-9 and TIMP-1 (0–200 ng/mL).

2.8. Immunohistochemical Analysis of MMP-2 and TIMP-2. Paraffin-embedded tissue of spinal cords from each group was cut into 5 μm thick sections. Immunohistochemistry was performed on these slices, using anti-rat antibodies for MMP-2 and TIMP-2 (all from Beijing TransGen Biotech Co.; Beijing, China). Sections were rinsed and incubated in nonbiotinylated goat anti-rabbit IgG secondary antibody. The chromophore product was developed using a Simple Stain DAB solution (Beijing TransGen Biotech Co., Beijing, China). The integral optical density (IOD) of positive cells in a restricted area was determined to represent the expressions of MMP-2 and TIMP-2 using Biosens Digital Imaging System v1.6.

2.9. Statistical Analysis. All data are presented as mean ± SD. Statistical analysis was performed with SPSS 16.0 (SPSS,

Table 1: Mean maximum clinical scores and body weight on day 17 p.i.

Groups	Body weight (g) (mean $\pm$ SD) ($n = 16$)
Naive control	224.38 ± 13.99
Vehicle-treated group	$154.63 \pm 10.83^{\triangle\triangle}$
MAT-L	$173.56 \pm 11.03^{**\#\#\lozenge}$
MAT-H	$182.31 \pm 10.12^{**\#\#\lozenge}$
DEX	$145.68 \pm 9.89^{*}$

$^{\triangle\triangle}P < 0.01$, compared to the naive group; $^{*}P < 0.05$ and $^{**}P < 0.01$, compared to vehicle-treated group; $^{\#\#}P < 0.01$, compared to DEX group; $^{\lozenge}P < 0.05$, comparison between MAT-L group and MAT-H group.

Chicago, USA). Multiple comparisons were performed using the Kruskal-Wallis test, or ANOVA, followed by the LSD-t test, as appropriate. A P value less than 0.05 was considered significant.

3. Results

3.1. MAT Treatment Alleviates Clinical Severity of EAE. In the vehicle-treated group, clinical decline typically started on day 10 p.i., while EAE onset for MAT-treated rats occurred on day 12 p.i. (low dose) and 13 p.i. (high dose) (Figure 1(b)). Compared to the vehicle-treated group, both MAT-treated groups exhibited significantly lower mean maximum clinical scores (Figure 1(c)) and body weight loss (Table 1). Treatment with DEX also delayed disease progression and reduced clinical scores compared to the vehicle-treated group ($P < 0.05$). There was no significant difference between animals treated with DEX and the two different doses of MAT.

3.2. Effect of MAT Treatment on CNS Histopathology. To assess EAE neuropathology, lumbar enlargements of spinal cord samples were examined using H&E and myelin staining (Figure 2). Perivascular cuffing with mononuclear cells and infiltration into CNS parenchyma were observed in the spinal cord of rats in the vehicle-treated group (Figure 2), while the extent of cellular infiltration was significantly decreased in the MAT-treated groups (both $P < 0.01$). Treatment with DEX showed a stronger inhibition in cellular infiltration than MAT-treated groups (both $P < 0.01$). Cellular infiltration was not observed in the naive group. Moreover, as shown in Figure 2, large areas of demyelination were observed in the vehicle-treated group, while MAT- and DEX-treated groups exhibited only a few areas of demyelination. No significant difference in demyelination was observed between MAT-H and DEX groups.

3.3. MAT Inhibits Evans Blue (EB) Leakage through the BBB. Destruction of the BBB is one of the important features of MS and EAE. We quantified the extravasation of EB dye into the brain as an indicator of BBB permeability. EB extravasation into the brain of vehicle-treated EAE rats was significantly higher than in naive brain ($P < 0.01$) (Figure 3). The content of EB was significantly decreased in MAT- and DEX-treated groups compared to the vehicle-treated group (all $P < 0.01$),

while a more profound decrease in EB content was observed in rats treated with MAT-H and DEX than with MAT-L ($P < 0.05$). No significant difference was observed between those treated with high doses of MAT and DEX.

3.4. MAT Protects Collagen IV and ZO-1 mRNA Expression. To determine the basement membrane disruption, we evaluated mRNA expression of collagen IV, a major component of the cerebral microvascular basement membrane, using RT-PCR analysis. As shown in Figure 4(a), the brightest band was exhibited in the naive group, while the faintest band was in the vehicle-treated group, consistent with quantitative analysis (Figure 4(c)). A dose-dependent increase in collagen IV expression was observed in MAT-treated groups. While the MAT-L-treated group showed lower collagen IV expression than the DEX group ($P < 0.01$), there was no significant difference between MAT-H and DEX groups.

We also assessed ZO-1 mRNA expression to determine TJ disruption. A similar pattern of mRNA expression of ZO-1 was observed for collagen IV (Figures 4(b) and 4(c)).

3.5. MAT Adjusts the Balance between MMP-9 and TIMP-1 in Serum. Serum concentration of MMP-9 was measured by ELISA. The amount of MMP-9 was dramatically increased in the vehicle-treated group compared to the naive group ($P < 0.01$) (Figure 5). MAT treatment largely reduced MMP-9 content compared to the vehicle-treated group (both $P < 0.01$), and the effect was dose dependent. Furthermore, a significantly lower amount of MMP-9 was observed in the DEX-treated group than in the MAT-treated groups ($P < 0.01$).

We also measured serum concentrations of TIMP-1 in the different groups. Figure 5 shows a significant decrease in TIMP-1 concentration in the vehicle-treated group compared to the naive group ($P < 0.01$). TIMP-1 serum levels in the MAT-treated groups were significantly higher than in the vehicle-treated group (both $P < 0.01$). While a low dose of MAT induced a lower serum TIMP-1 level than DEX ($P < 0.01$), a high dose of MAT increased the serum TIMP-1 level to a greater extent than DEX ($P < 0.01$).

3.6. MAT Regulates the Balance between MMP-2 and TIMP-2 in the CNS. To determine the MMP-2/TIMP-2 balance in the CNS, their expression in the spinal cord was measured by immunohistochemistry. As shown in Figure 6, MMP-2 expression was significantly increased in the vehicle-treated group over the naive group ($P < 0.01$). The differences in MMP-2 expression between vehicle-treated and the two MAT-treated groups were significant (both $P < 0.01$). Furthermore, the effect of DEX in decreasing MMP-2 content was stronger than low-dose MAT ($P < 0.05$), but there was no significant difference compared with the MAT-H group.

We then measured TIMP-2 content using the same method. As shown in Figure 6, TIMP-2 expression in the vehicle-treated group was significantly lower than in the naive group ($P < 0.01$) and MAT-treated groups ($P < 0.05$–0.01). A significantly lower amount was obtained in the MAT-L group than in the MAT-H group ($P < 0.05$). While the

| | Naive | Vehicle | MAT-L | MAT-H | DEX |
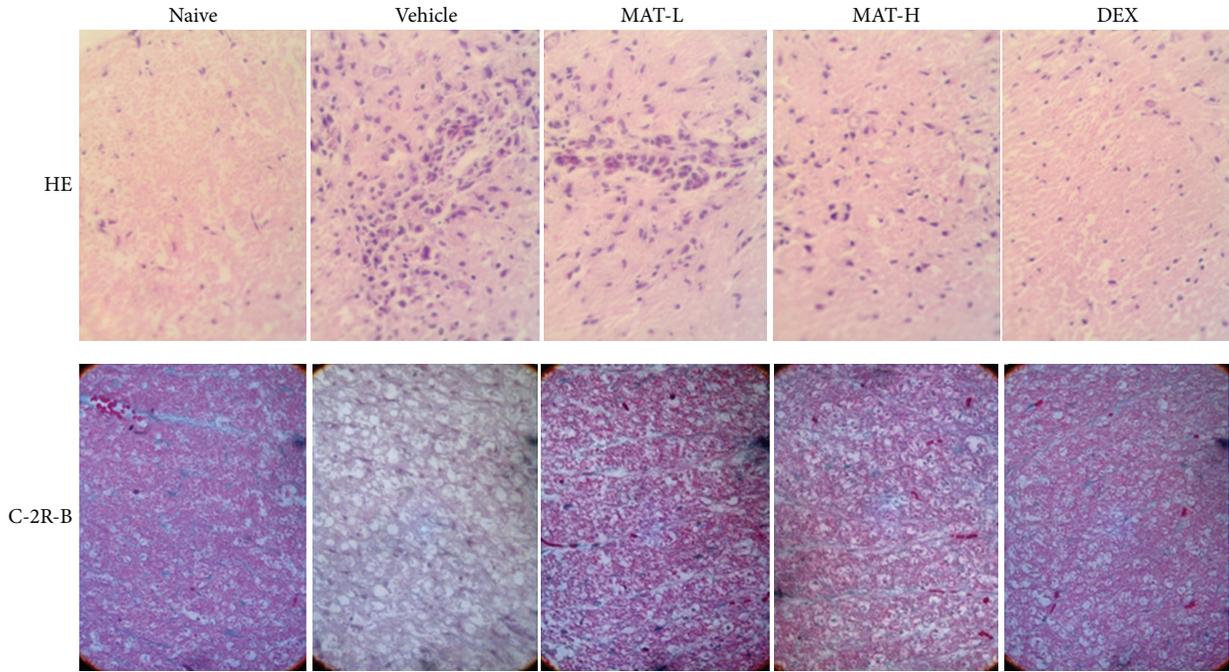
(a)
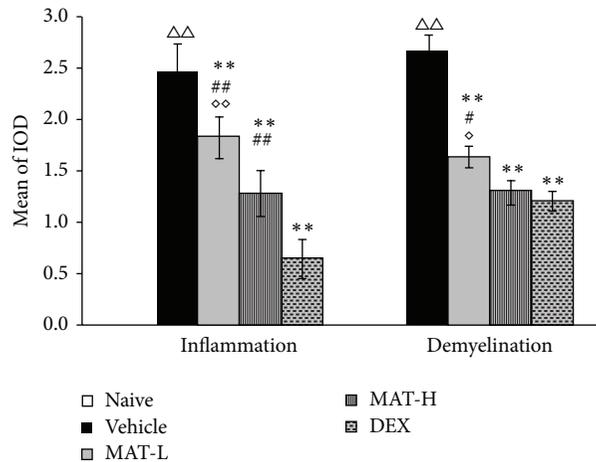
(b)

FIGURE 2: CNS infiltration and demyelination. At day 17 p.i., spinal cord lumbar enlargements were harvested and transverse sections were stained with H&E and C-2R-B. (a) Digital images were collected under bright-field setting using a ×40 objective. (b) Quantitative analysis. Mean values and SD are shown ($n = 8$ each group). $\triangle\triangle P < 0.01$, compared to naive group; $^{**}P < 0.01$, compared to vehicle-treated group; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$, compared to DEX group; $^{\lozenge}P < 0.05$ and $^{\lozenge\lozenge}P < 0.01$, comparison between MAT-L and MAT-H groups.

MAT-L-treated group showed lower TIMP-2 expression than the DEX-treated group ($P < 0.05$), there was no significant difference between MAT-H and DEX groups.

4. Discussion

Although administration of MAT reduced CNS inflammatory infiltration and demyelination, the effect of this natural compound on the BBB has not yet been studied. In the present study, we provide evidence that the therapeutic effect of MAT is, at least partially, through its strengthening of BBB integrity, its protection of the basement membrane as well as

TJ proteins, and its ability to regulate the balance between MMPs and TIMPs in both the periphery and CNS.

BBB destruction has been implicated in many CNS diseases, such as MS and stroke [4, 11]. In MS patients and in the EAE model, breakdown of the BBB is an early critical event, which is associated with the influx of inflammatory cells and, ultimately, with a poor outcome [3]. We thus quantified the extravasation of EB dye into the brain as an indicator of BBB permeability. Indeed, our study showed that EB leakage was markedly increased in the EAE model, which was associated with a decrease in collagen IV and ZO-1 expression in vehicle-treated rats compared to naive rats. MAT treatment largely
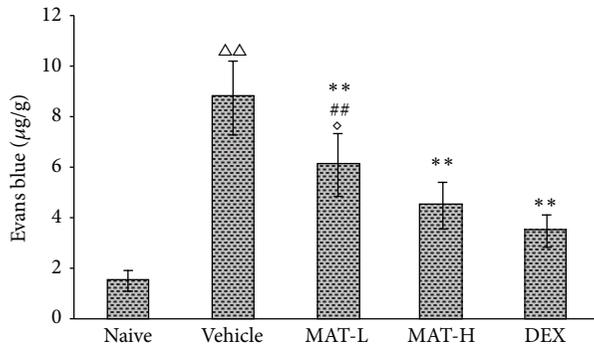
Figure 3: BBB integrity. Evans Blue was i.v. injected at day 17 p.i. and brains were harvested at 60 min to determine Evans Blue extravasation. Results are expressed as mean ± SD (n = 8 each group). $^{\triangle\triangle}P < 0.01$, compared to naive group; $^{**}P < 0.01$, compared to vehicle group; $^{\#\#}P < 0.01$, compared to DEX group; $^{\diamond}P < 0.05$, comparison between MAT-L and MAT-H groups.

and significantly decreased EB content in the brain compared with the vehicle group. Together, these results demonstrate that BBB integrity is compromised during the development of EAE and that MAT treatment protects the BBB.

BBB integrity depends on adequate structural support from the basement membranes, and collagen IV comprises up to 90% of total protein in the basement membrane [7]. In addition, collagen IV is critical for cell signaling by the interaction with various receptors and adhesion molecules, and its expression is therefore a marker of barrier damage and impairment [27]. A study by Lee et al. clearly shows that degradation of collagen IV has a role in the pathogenesis of BBB destruction and brain injury [28]. To date, it is not known whether decreased collagen IV contributes to BBB damage caused by EAE. We have shown in the present study that expression of collagen IV mRNA was significantly decreased in the vehicle-treated group compared to the naive group, and a dose-dependent increase of collagen IV mRNA expression was observed in MAT-treated groups. These results indicate that collagen IV degradation plays an important role in BBB disruption and that MAT treatment effectively preserves the content of collagen IV.

In the BBB, TJs are composed of large multiprotein complexes that mediate tight intercellular contacts among adjacent cells and play a critical role in maintaining BBB function by improving the barrier function at the endothelial level [29]. Previous publications have reported varying degrees of TJ pathology in EAE [30, 31]. Bennett et al. found that relocalization of ZO-1, which is a multidomain polypeptide required for the assembly of TJs [32], precedes disease onset and correlates with CNS infiltration in EAE [33]. Similar to these observations, our study found a decrease in ZO-1 mRNA expression in vehicle-treated group compared to the naive group and that ZO-1 mRNA levels were significantly improved with MAT treatment in a dose-dependent manner. These results are consistent with preserved collagen IV levels and indicate a protected BBB basement membrane.

In order to further study the mechanism of BBB protection induced by MAT treatment, we evaluated the activity

and balance of MMPs and TIMPs. MMPs, a group of zinc-containing endopeptidases, cleave most components of the basement membrane including fibronectin, laminin, proteoglycans, and collagen IV [34, 35]. It has been found that focal MMP-2 and MMP-9 activity is closely associated with the infiltrating T cells penetrating through the parenchymal basement membrane [5, 6]. Upregulation of MMP-2 and MMP-9 resulted in the degradation of TJs after focal ischemia/reperfusion, which can be reversed by MMP inhibition [36, 37]. In addition, a selective upregulation of MMP-9 in MS disease activity has been described [17]. Similarly, a significant increase in MMP-2 expression in the central canal of the cervical spinal cord is a sign of inflammation in acute EAE [38]. Furthermore, among the MMP family, MMP-9, together with MMP-2, is a member of the collagenase IV family, which has been implicated in the degradation of constituents of the basement membranes [15]. Targeting MMPs and chemokines has been considered an important therapeutic approach, alone or in combination with current medications, in enhancing their effect in neurological disorders such as MS [39]. In the present study, significant upregulation of MMP-9 in serum was observed in the vehicle-treated group compared to the naive group, and MAT treatment reduced the levels of MMP-9. Further, the loss of collagen IV and ZO-1 was reduced by blocking MMP-9 and MMP-2 in EAE. These studies suggest that overexpression of MMP-9 and MMP-2 in EAE could have been a causative agent in the reduced intensity of collagen IV and ZO-1; MAT treatment inhibition of MMP-9 and MMP-2 levels will preserve levels of collagen IV and ZO-1 and will thus be beneficial for BBB integrity.

The active forms of all MMPs are inhibited by a family of specific inhibitors, tissue inhibitors of metalloproteinases (TIMPs) [18, 19]. MMP-9 and MMP-2 are preferentially inhibited by TIMP-1 and TIMP-2, respectively, [17]. It has been found that normal homeostasis in the CNS requires a balance between MMPs and TIMPs, while an imbalance between these molecules is often associated with CNS pathology [40]. For example, radiation-induced brain injury is associated with an increased ratio between MMP-2 and TIMP-2 [21], and a significant increase in the MMP-9/TIMP-1 ratio also correlates with MS activity [17]. In our study, an imbalance between MMP-9 and TIMP-1 was observed in the vehicle-treated EAE group, with upregulation of MMP-9 and downregulation of TIMP-1; this was also the case for balance between MMP-2 and TIMP-2. We could speculate that overexpression of MMP-9/-2 in EAE was counterbalanced by MAT-mediated increase in TIMP-1/-2 expression, thus constituting a steady balance of MMPs and TIMPs that can be attributed to improved BBB function.

To further investigate the therapeutic effects of MAT on EAE, we compared MAT with DEX, a glucocorticoid, which was chosen as the positive control drug because of its strong ability to suppress inflammation. While MAT at a low dose showed a weaker effect than DEX, a comparable effect was observed between DEX and MAT-H groups, with a stronger effect of MAT in improving the TIMP-1 content in the serum. We believe that MAT would prove to be superior to DEX, whose long-term use carries with it the risk

(a)

(b)

(c)
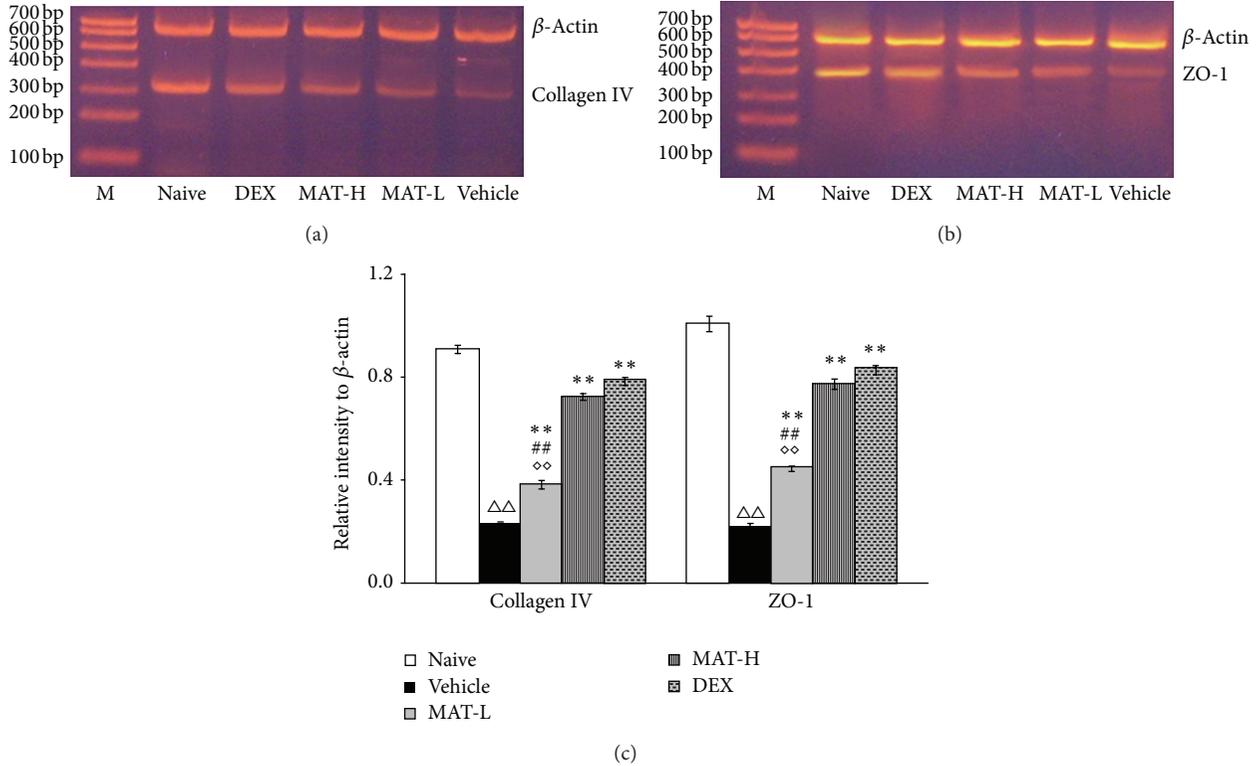
FIGURE 4: Collagen IV and ZO-1 mRNA expression. Spinal cords were harvested from treated and untreated EAE rats at day 17 p.i.; mRNA expression of collagen IV (a) and ZO-1 (b) was determined by RT-PCR, and an example of bands is shown. (c) Quantitative analysis. Results are expressed as mean ± SD ($n = 8$ each group). $^{\triangle\triangle}P < 0.01$, compared to naive group; $^{**}P < 0.01$, compared to vehicle group; $^{##}P < 0.01$, compared to DEX group; $^{\Diamond\Diamond}P < 0.01$, comparison between MAT-L and MAT-H groups.
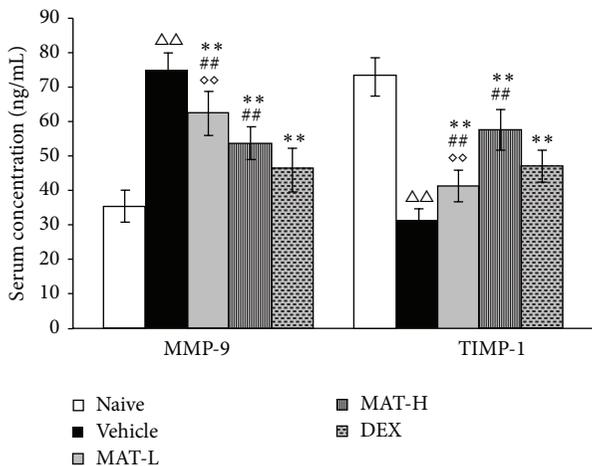
FIGURE 5: Serum concentration of MMP-9 and TIMP-1. At day 17 p.i., sera were harvested from treated and nontreated EAE rats, with sera from naive rats serving as control. MMP-9 and TIMP-1 production was determined by ELISA. Results are expressed as mean ± SD ($n = 8$ each group). $^{\triangle\triangle}P < 0.01$, compared to naive group; $^{**}P < 0.01$, compared to the vehicle group; $^{##}P < 0.01$, compared to the DEX group; $^{\Diamond\Diamond}P < 0.01$, comparison between MAT-L and MAT-H groups.

of side effects common to systemic glucocorticoids occurring over a relatively prolonged period. These side effects include hyperglycemia, hypertension, negative calcium balance, osteoporosis, weight gain, or even immunodeficiency [41, 42]. The extensive use of DEX in the treatment of severe acute respiratory syndrome (SARS) has often resulted in hypocortisolism and osteonecrosis of the femoral head, causing patients to lose the ability to work [43]. In contrast, patients with hepatitis B who used MAT for a long time showed significant therapeutic effect and good tolerance, with only minor side effects such as infrequent, transient dizziness and nausea. Whether long-term use of MAT would be safer and have fewer side effects than DEX needs further investigation.

In summary, our study demonstrates that improving BBB integrity is one of the mechanisms of MAT action in EAE therapy. This effect is at least partially through inhibiting activities of MMP-2/-9 and protecting the basement membranes and tight junction proteins, thus improving BBB integrity. As a result, inflammatory infiltration into the CNS is largely reduced, thereby protecting CNS tissues from proinflammatory cell/mediator-induced damage. While the process of immune cell extravasation is partially an endothelial cell-mediated process [44], whether MAT reduces this pathway of immune cell infiltration is not yet
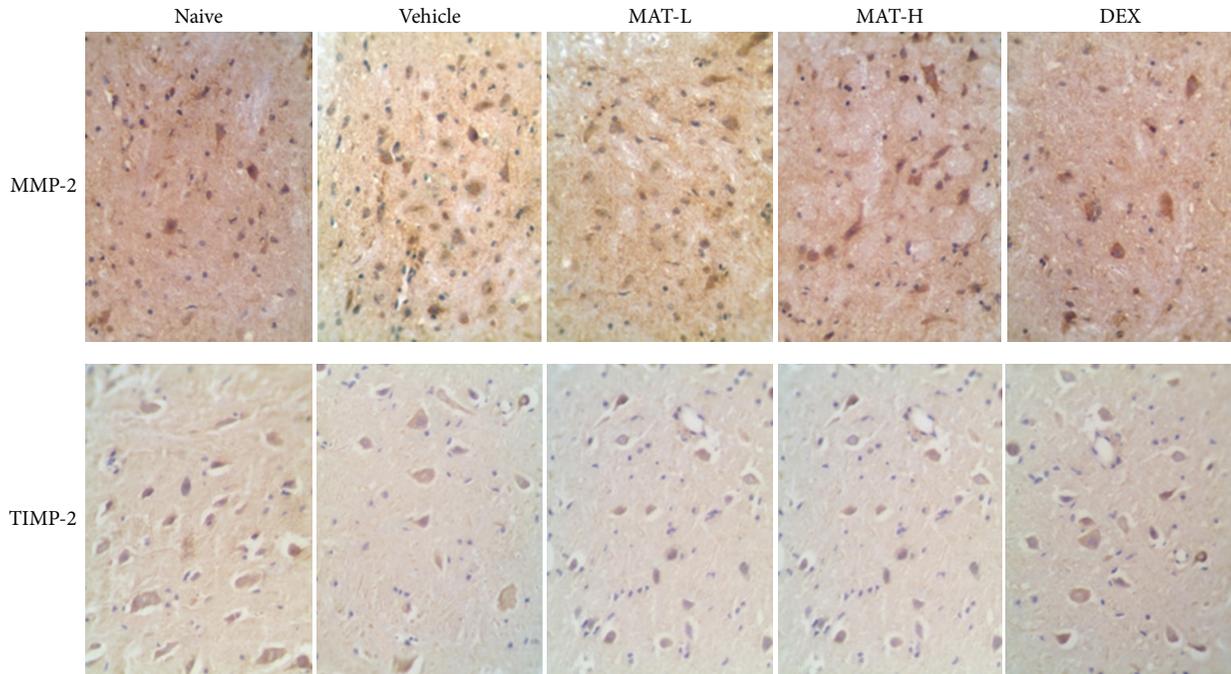
(a)
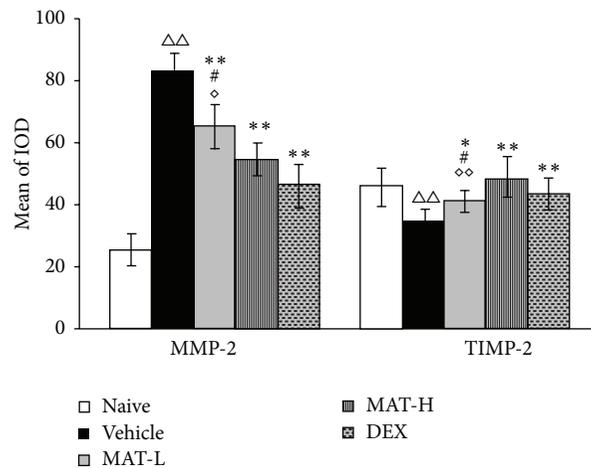
(b)

FIGURE 6: Immunohistochemistry of MMP-2 and TIMP-2. At day 17 p.i., spinal cords were harvested after extensive perfusion. (a) MMP-2 and TIMP-2 expression was determined by immunohistochemistry. Magnification: ×40. (b) Quantitative analyses. Values represent mean ± SD ($n = 8$ each group). $^{\triangle\triangle}P < 0.01$, compared to naive group; $^{*}P < 0.05$ and $^{**}P < 0.01$, compared to the vehicle group; $^{\#}P < 0.05$, compared to the DEX group; $^{\Diamond}P < 0.05$, comparison between MAT-L and MAT-H groups.

known. Nevertheless, results from the present study, together with the suppressive effect of MAT on Th1/Th17 cells [23] and its safety, suggest that MAT could qualify as an effective, alternative medication in MS therapy and that further study to test this possibility is warranted.

Conflict of Interests

None of the authors have a conflict of interests with any trademark mentioned in this paper.

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Review Article

Cytokines and Chemokines at the Crossroads of Neuroinflammation, Neurodegeneration, and Neuropathic Pain

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Cytokines and chemokines are proteins that coordinate the immune response throughout the body. The dysregulation of cytokines and chemokines is a central feature in the development of neuroinflammation, neurodegeneration, and demyelination both in the central and peripheral nervous systems and in conditions of neuropathic pain. Pathological states within the nervous system can lead to activation of microglia. The latter may mediate neuronal and glial cell injury and death through production of proinflammatory factors such as cytokines and chemokines. These then help to mobilize the adaptive immune response. Although inflammation may induce beneficial effects such as pathogen clearance and phagocytosis of apoptotic cells, uncontrolled inflammation can result in detrimental outcomes via the production of neurotoxic factors that exacerbate neurodegenerative pathology. In states of prolonged inflammation, continual activation and recruitment of effector cells can establish a feedback loop that perpetuates inflammation and ultimately results in neuronal injury. A critical balance between repair and proinflammatory factors determines the outcome of a neurodegenerative process. This review will focus on how cytokines and chemokines affect neuroinflammation and disease pathogenesis in bacterial meningitis and brain abscesses, Lyme neuroborreliosis, human immunodeficiency virus encephalitis, and neuropathic pain.

1. Introduction

Cytokines are a class of small proteins that act as signaling molecules at picomolar or nanomolar concentrations to regulate inflammation and modulate cellular activities such as growth, survival, and differentiation [1]. Cytokines are an exceptionally large and diverse group of pro- or anti-inflammatory factors that are grouped into families based upon their structural homology or that of their receptors. Chemokines are a group of secreted proteins within the cytokine family whose generic function is to induce cell migration [2, 3]. These "chemotactic cytokines" are involved in leukocyte chemoattraction and trafficking of immune cells to locations throughout the body. Chemokines belong to two categories based on their biological activity, namely, the maintenance of homeostasis and the induction of inflammation [4]. Homeostatic chemokines are involved in immune surveillance and navigation of cells through hematopoiesis and are typically expressed constitutively. Inflammatory chemokines on the other hand are produced during infections or as a response to an inflammatory stimulus and facilitate an immune response by targeting cells of the innate and adaptive immune system. The binding of a cytokine or chemokine ligand to its cognate receptor results in the activation of the receptor, which in turn triggers a cascade of signaling events that regulate various cellular functions such as cell adhesion, phagocytosis, cytokine secretion, cell activation, cell proliferation, cell survival and cell death, apoptosis, angiogenesis, and proliferation [5].

In the field of neuroimmunology, the classical view that regarded the central nervous system (CNS) as an immune-privileged site by virtue of its shield, the blood brain barrier (BBB), has evolved to a view of significant CNS-immune system interactions [6]. Cytokines and chemokines are involved in the regulation of CNS-immune system interactions besides

being important for the coordination of immune responses throughout the body. They are produced primarily not only by white blood cells or leukocytes but also by a variety of other cells as a response to various stimuli under both pathological and physiological conditions. In the nervous system, cytokines and chemokines function as neuromodulators and regulate neurodevelopment, neuroinflammation, and synaptic transmission. Cytokines and chemokines are crucial to the brain's immune function serving to maintain immune surveillance, facilitate leukocyte traffic, and recruit other inflammatory factors [7]. Upon stimulation by pathogens or abnormal cells, immune cells as well as cells of the nervous system such as microglia (the resident macrophages of the brain), astrocytes, oligodendrocytes, the myelinating cells of the CNS, and Schwann cells in the peripheral nervous system (PNS), endothelial cells of the brain microvasculature, and even neurons can release cytokines and chemokines as well as respond to them by way of cytokine and chemokine receptors [8–10]. Neuroinflammatory processes significantly affect both health and disease of the nervous system by regulating the development, maintenance, and sustenance of brain cells and their connections. In the steady state, microglia protect the nervous system by acting as scavengers of debris and microbial pathogens and by regulating the innate and adaptive immune responses. Pathological states within the nervous system including injury, ischemic stroke [11], and infection [12] can lead to activation of microglia. This in turn can cause release of inflammatory molecules that trigger astrocytes and cells of the immune system to respond to the injury [13]. In the disease state, activated microglia mediate neuronal and glial cell injury and death through production of proinflammatory factors like cytokines and chemokines, glutamate, and reactive oxygen species among others and help mobilize the adaptive immune response and cell chemotaxis, leading to transendothelial migration of immune cells across the BBB and even perpetuation of neuronal damage [14]. The central role of microglia in orchestrating neuroinflammation is described in Figure 1.

In response to injury, neurons produce adhesion molecules and trophic factors that recruit microglial cells and astrocytes. The latter can participate in the ongoing process of damage and repair. In addition to glial cells, the microvasculature also participates in this process. Neurodegeneration is concomitant with astrogliosis, microgliosis, and microvasculature remodeling. Though the trophic factors released initially by astrocytes during astrogliosis aid in tissue repair, these factors amplify the inflammatory response, augment vascular permeability, and result in increased microglial activation and release of more cytokines and chemokines. In states of prolonged inflammation, continual activation and recruitment of effector cells can establish a feedback loop that perpetuates inflammation and ultimately results in neuronal injury [14]. Thus, a critical balance between repair and proinflammatory factors determines the rate of progression and outcome of a neurodegenerative process.

Understanding the role of proinflammatory cytokines in neurodegenerative diseases is complicated by the cytokines' dual roles in neuroprotection and neurodegeneration. For example, IL-6 has dual roles in brain injury and disease.

It is produced during reactive astrogliosis as a response to neuronal damage, acting as a neurotrophin promoting neuronal survival, while elevated levels of IL-6 have also been adversely associated with several brain diseases [15].

Some cytokines like IL-1β and TNF induce neurotoxicity through elevated glutamate production that results in neuronal excitotoxic death [16]. The inactivation of IL-1β and TNF using neutralizing antibodies significantly reduced neuronal death in SK-N-SH cells induced by West Nile Virus [17]. Neuroinflammation and both cytotoxic and vasogenic edema were reduced in IL-1 type 1 receptor-deficient mice conferring neuroprotection in stroke [18]. IL-1β also promotes oligodendrocyte death through glutamate excitotoxicity [19]. IL-1β and TNF can cause death of oligodendrocytes in a calcium dependent manner [20]. Deletion of the TNF gene ameliorates neurodegeneration in Sandhoff disease (SD), a lysosomal storage disorder [21]. TNF acts as a neurodegenerative cytokine mediating astrogliosis and neuronal cell death in SD, suggesting TNF as a potential therapeutic target to attenuate neuropathogenesis [21]. On the contrary, aggravation of experimental autoimmune neuritis has been observed in TNF-α receptor 1 deficient-mice signifying an anti-inflammatory role for TNF in this mouse model [22]. TNF has been implicated in both neuronal death and survival and the level and time of expression determine its final effect on CNS damage or protection [23].

Evidence is emerging that chemokines play a role in the physiology of the nervous system, including neuronal migration, cell proliferation, and synaptic activity, besides mediating neuroinflammation. Chemokines are implicated in many diseases of the nervous system. Although their primary role is to induce inflammation through the recruitment of leukocytes by their chemotactic activity, they may also have direct effects on neuronal cells. Chemokines and their receptors are among the key players responsible for communication between neurons and inflammatory cells, and this crosstalk is crucial for normal neurological functioning. Evidence of major roles for chemokines and their receptors in diseases of the brain is accumulating and this system is a potential target for treatment of neurodegenerative diseases [24].

Chemokines may induce neuronal death directly through the activation of neuronal chemokine receptors or indirectly through the activation of microglial killing mechanisms. In addition, some chemokines have neuroprotective roles and function as pro- or anti-inflammatory mediators. For example, induction of neuronal MCP-1/CCL2 during mild impairment of oxidative metabolism caused by microglial recruitment/activation exacerbated neurodegeneration in thiamine-deficiency- (TD-) induced neuronal death, while CCL2-knockout (KO) mice were resistant to TD-induced neuronal death, suggesting that the chemokine CCL2 mediates microglial recruitment and neurodegeneration in this model [25]. However, in another system, CCL2 protected neurons from the toxic effects of glutamate and HIV-tat-induced apoptosis [26]. Interestingly, MCP-1-deficient mice showed reduced neuroinflammatory responses and increased peripheral inflammatory responses to peripheral endotoxin insult. These data demonstrate an important role for MCP-1
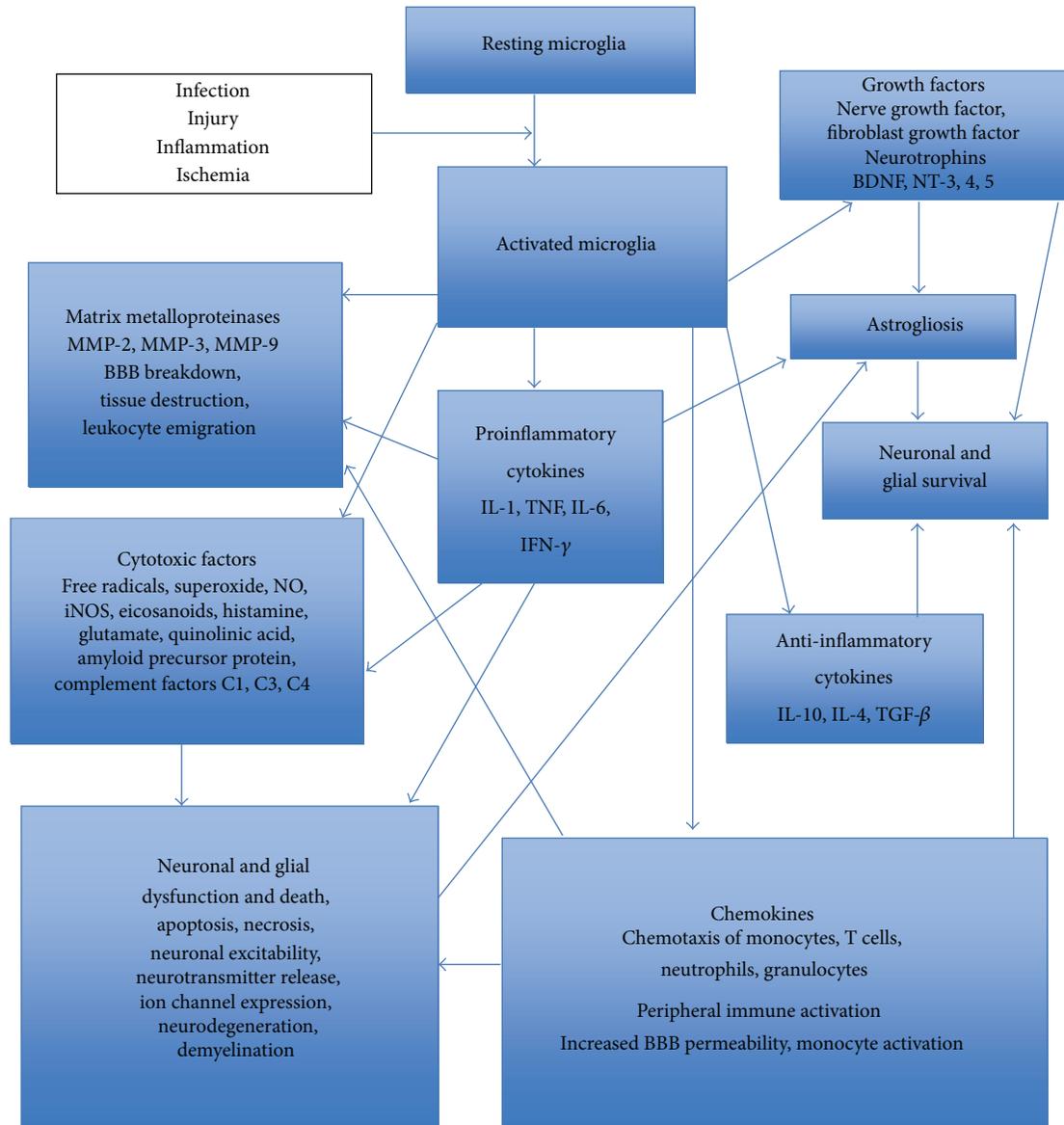
FIGURE 1: Central role of microglia in neuroinflammation.

in regulation of brain inflammation after peripheral endotoxemia [27].

The overexpression of CXCL10 or interferon gamma-induced protein 10 (IP-10) has been observed in several neurodegenerative diseases including multiple sclerosis (MS), Parkinson's disease (PD) HIV-associated dementia, and Alzheimer's disease (AD) [28–31]. CXCL10 elicits apoptosis in fetal neurons by elevating intracellular calcium levels [32]. On the contrary, the signaling of the neuronal chemokine fractalkine (CX3CL-1) and its receptor CX3CR1 has been shown to be neuroprotective, as they reduce the levels of neurotoxic substances like TNF and nitric oxide in activated microglia during neuroinflammation [33].

The chemokine IL-8 (CXCL8) that regulates neutrophil migration by signaling through the CXCR2 receptor is markedly elevated by brain injury and is associated with the propagation of secondary damage. Evaluating the function of CXCR2 in posttraumatic inflammation and secondary degeneration by examining Cxcr2-deficient (Cxcr2−/−) mice showed reduced tissue damage and neuronal loss in Cxcr2−/− mice compared to wild-type controls [34]. CXCR1, the receptor for MIP-2 (CXCL2) and CXCR2, has also been implicated in contributing directly to motor-neuron degeneration [35].

The chemokine CXCL1 or GRO1 that is upregulated in brain endothelium in the presence of IL-6 has been identified as a key regulator of granulocyte recruitment into the CNS. Though granulocytes generally exert a protective role in the CNS, they have been shown to be detrimental in experimental autoimmune encephalitis (EAE), the most common model of MS. Administering anti-CXCL1 antibodies attenuated EAE severity suggesting CXCL1 to be a new potential target for the treatment of neuroinflammatory conditions like MS [36].

The chemokine CXCL12 or stromal cell-derived factor 1 (SDF-1) is strongly chemotactic for lymphocytes and it modulates neurotransmission, neurotoxicity, and neuroglial interactions [37]. Growing evidence implicates enhanced expression of CXCL12 and its receptor CXCR4 in the pathogenesis of CNS disorders such as HIV-associated encephalopathy, brain tumor, stroke and MS, making them promising targets for pharmacological intervention [38]. CXCL12/CXCR4 have been shown to promote apoptotic death of dopaminergic neurons in a mouse model of PD [39]. CXCL12/CXCR4 have been suggested to be markers to grade CNS glioblastoma tumor progression. In glioblastoma, a CXCR4 antagonist (AMD3100) showed an inhibition of tumor growth [40]. Thus, several studies using cytokine/chemokine receptor antagonists and deletion mutant mice have provided exciting findings establishing a central role for cytokines and chemokines in mediating neuroinflammation and neurodegeneration and cytokines/chemokines and their receptors represent interesting therapeutic targets in this context.

Immune activation in the nervous system is associated with pathological conditions such as bacterial and viral infections, autoimmune diseases, and inflammatory neurodegenerative disorders including AD, PD, amyotrophic lateral sclerosis, MS [41–44], and Lyme neuroborreliosis (LNB) [45]. Peripheral neuropathies such as Guillain-Barré syndrome [46], PNS Lyme neuroborreliosis [47], demyelinating polyradiculoneuropathies [48], and conditions of neuropathic pain [10, 49] are also accompanied by inflammation. One underlying similarity in all these disease states is the cytokine and chemokine-driven inflammatory response. The dysregulation of cytokines and chemokines is a central feature in the development of neuroinflammation, neurodegeneration and demyelination in the CNS [43, 44], neuritis and axonal degeneration in the PNS [9], and conditions of neuropathic pain [10, 49]. Understanding the involvement of cytokines and chemokines in the pathogenesis of nervous system disorders is relevant for understanding brain pathophysiology and may lead to the development of targeted therapies to treat neurodegenerative diseases. This review will focus on how cytokines and chemokines affect neuroinflammation and disease pathogenesis in bacterial meningitis and brain abscesses, Lyme neuroborreliosis, human immunodeficiency virus encephalitis (HIVE), and neuropathic pain.

2. Cytokines and Chemokines in Bacterial Meningitis and Brain Abscesses

Bacterial meningitis is among the top ten causes of death due to infectious agents worldwide. The major meningeal bacterial pathogens are *Streptococcus pneumoniae*, *Neisseria meningitides*, and *Hemophilus influenzae*, although other organisms are capable of causing disease in humans in an age-specific manner [50]. About 50% of patients surviving the infection present with neurological deficits [51]. The pathogen gains access to the bloodstream, penetrates the BBB, and replicates in the subarachnoid space. Immune cells from the peripheral circulation are attracted into the infected subarachnoid space by inflammatory mediators that are produced initially by ependymal cells, meningeal macrophages,

and choroid plexus epithelium, followed by local microglia and recently emigrated leukocytes [52–54]. The pathological manifestations of meningitis include increased intracranial pressure, intense brain edema, impairments in cerebrospinal fluid (CSF) flow, seizures, and alterations in cerebral blood flow that may result in focal areas of ischemia and necrosis. During bacterial meningitis, the antibacterial response elicited by the host can be detrimental to neurons and glia in the CNS, due to the toxic effects of cytokines, chemokines, proteolytic enzymes, and oxidants produced locally at the site of infection, in addition to the direct damage caused by pathogens [55].

2.1. Streptococcus pneumoniae Meningitis. *S. pneumoniae* is the most frequent cause of bacterial meningitis [56, 57]. Effects from meningitis can range from memory deficits, hearing loss, hydrocephalus, cerebral palsy, and seizures. Since the pneumococci can cross the BBB, microglia may respond directly to intact bacteria or to pneumococcal cell wall antigens and produce a wide array of inflammatory mediators including TNF, IL-6, IL-12, keratinocyte-derived chemokine (CXCL1/KC), CCL2/MCP-1,CCL3/MIP-1α, CXCL2/MIP-2, and CCL5/RANTES, as well as soluble TNF-α receptor II, a TNF antagonist [58, 59]. The production of these inflammatory mediators is associated with the activation of the extracellular signal-regulated protein kinases ERK-1 and ERK-2 via a MAPK intracellular signaling pathway [58, 59].

The microglial-derived cytokine and chemokine profile represents a double-edged sword since it is effective at eliciting leukocyte recruitment into the CNS for the purpose of antibacterial defenses but at the same time can also contribute to inflammatory mediator-induced neuronal damage by apoptosis triggered, in part, by the inflammatory process via caspase activation [60]. This suggests that strategies to check microglial activation at a point where inflammation is no longer beneficial could reduce damage to surrounding normal parenchyma as a result of bystander destruction. It is suggested that neuronal damage in bacterial meningitis is caused by the dual effects of an overwhelming inflammatory response and the direct effects of bacterial toxins [61]. It is proposed that brain damage in bacterial meningitis leading to long-term neurologic sequelae and death involves several mechanisms. Bacterial invasion and the release of bacterial compounds promote inflammation, invasion of leukocytes, and stimulation of microglia. Leukocytes, macrophages, and microglia release free radicals, proteases, cytokines, and excitatory amino acids, eventually leading to energy failure and cell death. In addition, vasculitis, focal ischemia, and brain edema subsequent to an increase in CSF outflow resistance, breakdown of the BBB, and swelling of necrotic cells cause secondary brain damage.

2.2. Cytokines in the Pathogenesis of Pneumococcal Meningitis. The early response cytokines TNF, IL-1, and IL-6 are produced after pneumococcal recognition, which in turn induce upregulation of several adhesion factors on the vascular endothelium, mediating leukocyte influx [62]. An experimental rabbit model of pneumococcal meningitis has shown that the outcome of bacterial meningitis is related to

the severity of inflammation in the subarachnoid space and that outcome can be improved by modulation of the inflammatory response [63]. Homologous antibodies to TNF, IL-1α and IL-1β inhibited leukocytosis and brain edema and moderately decreased BBB permeability in this model of meningitis [64]. A mouse model of *S. pneumoniae* meningitis that mimics several features of human disease describing meningeal inflammation and neuronal damage [65] and an infant mouse model of brain damage in pneumococcal meningitis that exhibits neuronal brain injury in the cortex and hippocampus that reflect the histomorphological findings in the human disease have been established [66]. The infant rat model of pneumococcal meningitis has also been very useful to study the pathogenesis of the disease [67, 68].

The use of KO mice has offered new insights into the role of cytokines involved in the inflammatory cascade during pneumococcal meningitis. Increased mortality and spatial memory deficits in TNF-α-deficient mice with experimental pneumococcal meningitis were observed suggesting that TNF plays a role in inflammation and hippocampal injury in bacterial meningitis [69]. Patients with pneumococcal meningitis show increased CSF-TNF-α, which correlates with severity of BBB disruption, disease severity, and neurological sequelae [70].

IL-1 is an important proinflammatory cytokine, which is upregulated in brain tissue after the induction of meningitis. Mortality was significantly higher and appeared earlier in the course of the disease in IL-1R ($-/-$) mice demonstrating that endogenous IL-1 is required for an adequate host defense in pneumococcal meningitis [71]. IL-18 gene-deficient mice showed enhanced defense and reduced inflammation during pneumococcal meningitis suggesting that endogenous IL-18 contributes to a detrimental inflammatory response during pneumococcal meningitis and that elimination of IL-18 may improve the outcome of this disease [72].

The anti-inflammatory cytokine IL-10 has been implicated in playing a role in modulating the immune response by downregulating TNF, IL-6, and keratinocyte-derived chemokine (KC), thereby reducing CSF pleocytosis in pneumococcal meningitis [71]. IL-10 has been shown to repress sepsis-associated hippocampal neuronal damage as a result of pneumococcal sepsis in mice overexpressing IL-10 [73]. Also, intravenously administered recombinant IL-10 reduced the levels of CSF pleocytosis, cerebral edema, and intracranial pressure in a rat model of pneumococcal meningitis [74]. In mice with *S. pneumoniae*-induced meningitis, a deletion of TGF-β receptor II on leukocytes is found to enhance recruitment of neutrophils to the site of infection and to promote bacterial clearance. The improved host defense against *S. pneumoniae* was associated with an almost complete prevention of meningitis-induced vasculitis, a major intracranial complication leading to brain damage. The data show that endogenous TGF-β suppresses host defense against pneumococcal infection in the CNS [75]. Activin A, a member of the TGF-β superfamily and a neuroprotectant that is expressed constitutively in the CSF, has been shown to be upregulated in patients during bacterial meningitis [76, 77]. Cotreatment with activin A and LPS showed increased microglial proliferation and negative regulation of NO,

IL-1β, IL-6, and TNF in *in vitro*-cultured murine microglia [77].

2.3. Chemokines in the Pathogenesis of Pneumococcal Meningitis.
Multiple chemokines have been reported to be upregulated in the CSF of patients with pneumococcal meningitis including CCL15, CXCL7, MIF, CCL8, CCL18, CCL20, CXCL5, CXCL-1, CXCL-8, CCL2, CCL3, and CCL4 [78–81]. In animal models of pneumococcal meningitis, additional chemokines have been identified by protein arrays for brain tissues, including CCL9, CXCL-2, XCL-1, CCL-1, CCL11, CCL12, CCL24, CCL25, CXCL4, CXCL10, CXCL12, CXCL13, and CXCL13 [82].

IL-8 (CXCL-8 was found to be chemotactic for neutrophils in the CSF of patients with bacterial meningitis [79]. IL-8 appears to regulate CSF pleocytosis in pneumococcal meningitis from the systemic compartment, similar to that seen for TNF, IL-10, and TGF-β [83]. Both MIP-1 (CCL3) and MIP-2 (CCL3) are produced by immune cells resident in the brain and attract monocytes and neutrophils from the bloodstream into the CSF in acute bacterial meningitis [84]. *In vitro*, antibodies against CCL2, CCL3, and CCL4 inhibited monocyte chemotactic properties of CSF from patients with pneumococcal meningitis [79]. The intracisternal inoculation of recombinant CCL3 and CCL4 induced BBB disruption, CSF leukocytosis, and cerebral edema in a rabbit model of pneumococcal meningitis [64]. Of the CXCL chemokines, ENA-78 (CXCL5) was found to be upregulated in patients with bacterial meningitis and exhibited neutrophil chemotactic properties together with IL-8 [80]. A study that evaluated the global response of the BBB to *S. pneumoniae* infection and the specific role of neuraminidase A (NanA), a pneumococcal protein described to promote CNS trophism, revealed that NanA was necessary and sufficient to activate host chemokine induction including IL-8, CXCL-1, and CXCL-2 [85]. In summary multiple chemokines have been reported to be upregulated in pneumococcal meningitis that primarily have a role in attracting leukocytes to the CSF, though the roles of many chemokines in the pathogenesis of the disease have not yet been investigated [86].

2.4. Staphylococcus aureus and Brain Abscesses.
Abscesses in brain parenchyma develop as a consequence of local spread of pyogenic bacteria from the paranasal sinuses, middle ear, or oral cavity, via hematogenous dissemination from a systemic infection or by directly penetrating trauma to the head [87–92]. The most common etiologic agent of brain abscesses in humans is *S. aureus*, besides Streptococci [88]. These infections are characterized by extensive edema and tissue necrosis [89]. The activation of resident microglia is a hallmark of infection [90], in addition to the sequential progression to necrosis during brain abscess evolution. Microglial and astrocyte activation is evident immediately following the entry of bacteria into the CNS parenchyma and persists throughout abscess development in a mouse model [91]. The ensuing abscess formed at the site of infection may result in inflammation accompanied by edema, neuronal toxicity, seizures, and long-term cognitive loss [92]. This murine model has demonstrated that *S. aureus* not only induces

brain abscesses but also elicits rapid and sustained expression of numerous proinflammatory cytokines and chemokines including IL-1β, TNF, IL-12 p40, CXCL2, CCL2, CCL3, and CCL4 [93–95]. Leukocyte recruitment elicited by microglia into the infected CNS facilitates bacterial clearance during abscess development. Microglia also exert *S. aureus* bactericidal activity. The organism is a potent inducer of numerous inflammatory molecules in microglia such as TNF, IL-1β, and CXCL1, among others [96, 97]. The necrotic damage associated with brain abscesses and other CNS infections is accompanied by release of endogenous host molecules that could potentially exacerbate parenchymal necrosis in addition to that mediated by unchecked microglial activation.

Knowledge of the staging of brain abscess in humans is based on findings of CT and MRI scans [98]. During the last decade, an experimental brain abscess model in rats and mice has been established by direct intracerebral injection of live *S. aureus*, [90, 92, 99, 100]. Rodent models mimic accurately the natural course of brain abscess development in humans and have been investigated intensely to understand the mechanisms in disease pathogenesis and the possible treatment modalities.

Brain abscess is typified by a sequential series of pathological changes that have been elucidated in experimental rodent models [90, 92, 99–101]. Briefly, the early stage cerebritis occurs from day 1 to day 3 and is marked by neutrophil accumulation, tissue necrosis, and edema. Astrocyte and microglial activation are seen at this stage and persist throughout abscess development, accompanied by the induction of proinflammatory cytokines and chemokines [90, 94]. From day 4 to day 9 (intermediate or late cerebritis), predominant macrophage and lymphocyte infiltrates are commonly seen. The last or capsule stage is seen from day 10 onwards and is characterized by the formation of a well-vascularized abscess wall that in turn helps to sequester the lesion and protects the surrounding brain parenchyma from additional damage.

However, the immune response that is essential for abscess formation also destroys surrounding normal brain tissue. There is a prolonged expression of IL-1β, TNF, and macrophage inflammatory protein-2 (MIP-2/CXCL2), concomitant with a chronic disruption of the BBB in mice with *S. aureus*-induced brain abscess. These changes correlated with the continued presence of infiltrating neutrophils and macrophages/microglia. These observations suggest that the excessive tissue damage that often results from brain abscess may be mediated, in part, by the perpetuation of antibacterial immune responses that are not down-regulated in a timely manner [91]. Similarly, human brain abscess lesions have been found to encompass a large area of the brain, often spreading well beyond the initial focus of infection [102].

On the other hand, cytokines like IL-1β, TNF, and IL-6 may exert beneficial effects on the establishment of host antibacterial immune responses. A study that examined the relative importance of IL-1β, TNF, and IL-6 in experimental brain abscess using cytokine KO mice showed that IL-1 and TNF play a major role in directing the ensuing antibacterial response, as bacterial burdens were significantly higher in both IL-1 and TNF-α-KO mice compared to wild-type mice

which correlated with enhanced mortality rates in KO mice [91].

Neutrophils that are the major peripheral cell infiltrate associated with brain abscesses are the source of proinflammatory cytokines such as TNF that serve to amplify the antibacterial immune response [103]. Neutrophils can also exert bactericidal activity through the production of reactive oxygen intermediates and nitrogen intermediates and hydrolytic enzymes that can directly destroy bacteria; however, the continuous release of cytokines and bactericidal products by neutrophils can also contribute to tissue damage [103]. CXCR2 ligands, namely, MIP2 (CXCL2) and KC (CXCL2) are the major ligands required for chemotactic signaling of neutrophils into brain abscesses as demonstrated by CXCR2 KO mice studies [92]. Impaired neutrophil influx into evolving brain abscesses in antibody-mediated neutrophil-depleted mice and CXCR2 KO mice resulted in exacerbated disease accompanied by elevated bacterial burdens compared to wild-type mice [92, 104]. In addition, chemokines such as CCL1, CCL2, CCL3, and CCL4 were also detected in evolving brain abscesses that most probably contribute to the influx of lymphocytes and monocytes and the establishment of the adaptive immune response [92].

Using a minocycline-resistant strain of *S. aureus* to dissect the antibiotic's bacteriostatic versus immune modulatory effects in a mouse experimental brain abscess model, minocycline was found to significantly reduce mortality rates within the first 24 hours following bacterial exposure [105]. This protection was associated with a transient decrease in the expression of several proinflammatory mediators, including IL-1β and CCL2. Minocycline was also capable of protecting the brain parenchyma from necrotic damage as evidenced by significantly smaller abscesses in minocycline-treated mice. In addition, minocycline exerted anti-inflammatory effects when administered as late as 3 days following *S. aureus* infection, which correlated with a significant decrease in brain abscess size. Finally, minocycline was capable of partially attenuating *S. aureus*-dependent microglial and astrocyte activation. This study suggests that minocycline may afford additional therapeutic benefits extending beyond its antimicrobial activity for the treatment of CNS infectious diseases typified by a pathogenic inflammatory component through its ability to balance beneficial versus detrimental inflammation.

3. Cytokines and Chemokines in Lyme Neuroborreliosis

Lyme borreliosis the most frequently reported vector-borne disease in the USA is caused by the spirochete *Borrelia burgdorferi (Bb)* [106]. It is transmitted through *Ixodes* ticks and is also prevalent in Europe and Asia [106, 107]. Lyme neuroborreliosis (LNB), the form of Lyme disease that affects the nervous system, manifests in about 15% of Lyme disease patients and affects both CNS and PNS [108–110]. Patients with CNS involvement complain of severe headaches, flu-like symptoms, fatigue, memory loss, learning disability, and/or depression. Infection of the PNS with *Bb* may result in facial

nerve paralysis or palsy, pain in the back and limbs, and movement disorders.

Clinically, LNB may manifest as meningitis typically characterized by lymphocytic pleocytosis in the CSF, meningoradiculitis (a.k.a. Bannwarth's syndrome), cranial neuritis, encephalopathy, peripheral neuropathy, and, less commonly, encephalitis and encephalomyelitis. Radiculitis, or inflammation in the dorsal roots, is the most common manifestation of untreated Lyme borreliosis in humans [110]. LNB patients may also experience a wide array of neurological symptoms as a result of white matter inflammation that results in a subacute MS-like manifestation [111, 112]. Perivascular and vascular inflammatory processes may also occur in CNS LNB and several case reports of seizures or stroke have been attributed to neurologic Lyme disease [113–115].

Reports from human cases of LNB often include lymphocyte and plasma cell infiltration in the meninges and perivascularly in the nerve roots, dorsal root ganglia (DRG), and demyelination in the brain and spinal cord [113, 116–120]. Typically, PNS-Lyme disease is associated with transverse myelitis and patchy multifocal axonal loss with epineural perivascular inflammatory infiltrates or perineuritis [121–125]. The primary findings of axonal degeneration and regeneration and multifocal nerve lesions showing perivascular inflammatory cellular infiltrates have been documented in almost all patients with Lyme-associated peripheral neuropathy [123–127]. The results of these studies suggest that immune mediated neuronal and glial cell damage could be involved in the neuropathogenesis of LNB.

It is suggested that adherence of the spirochete to the endothelium lining of blood vessel walls leads to the release of inflammatory mediators [128]. This could in turn alter the permeability of the BBB and ensue entry of *Bb* into the CNS [128, 129]. The perivascular mononuclear cell infiltrates observed in the cerebral cortex during *Bb* infection consist predominantly of T-helper cells [130] and are associated with a focal increase in microglial cells and infiltration of lymphocytes and plasma cells in the leptomeninges [131].

Elevated levels of the proinflammatory cytokines IL-6, IL-8, IL-12, IL-18, and interferon γ [71–74] and the chemokines interferon-inducible T-cell chemoattractant (I-TAC), CCL2, CXCL-11, and CXCL13 [132–139] have been reported in the CSF of patients with LNB. The amount of IL-6 in human serum and CSF has been shown to correlate with disease activity in neurologic Lyme disease [132]. The chemokine CXCL13, which is known to attract B-lymphocytes, is also elevated in other instances of neuroinflammation [140]. CXCL13 expression in the CSF precedes the intrathecal production of *Bb*-specific antibodies [141] and may account for the high proportion of B-lymphocytes and plasma cells in the CSF of LNB patients, suggesting a role for the humoral immune response in Lyme neuroborreliosis [142].

Increased production of the neuromodulator quinolinic acid, an excitotoxin and N-methyl-D aspartate (NMDA) agonist, has been demonstrated in the CSF of patients with neurologic Lyme disease [143]. As the NMDA receptor mediates synaptic function and is involved in learning, memory, and synaptic plasticity [144], its dysregulation mediated by *Bb*-induced inflammation may contribute to the neurologic and cognitive deficits seen in many Lyme disease patients by mechanisms such as glutamate-mediated excitotoxicity [145].

The rhesus macaque is the preferred animal model for studying neurologic Lyme disease, as it exhibits most of the signs of Lyme disease seen in humans, both in the PNS and CNS [146, 147]. Using the monkey model, investigators have been able to examine the effect of *Bb* infection on neural tissue and its relationship to the adaptive and acquired immune responses. Since *Bb* itself does not produce any known endotoxin [148], damage to neural cells may occur in part due to bacterial lipoproteins that are present on the spirochetal surface or are released or shed by live or dead organisms. Lipidated outer-surface protein A (L-OspA), a prototype *Bb* lipoprotein, has been shown to induce IL-6, cell proliferation, and concomitant apoptosis in rhesus astrocytes *in vitro* [149, 150]. *Bb*-infected rhesus monkeys also showed astrogliosis in the frontal cortex [149]. In the presence of *Bb*, primary cultures of astrocytes or microglia have been shown to produce IL-6, IL-8, and the macrophage inflammatory proteins CCL3 and CCL4 [151]. Human neurons cocultured with *Bb* and rhesus microglia undergo apoptosis in the presence of proinflammatory mediators chiefly produced by the microglia [152].

In an *ex vivo* stimulation of monkey brain frontal cortex tissue explants with live *Bb*, IL-6, IL-8, IL-1β, and CXCL13 were visualized in glial cells, with concomitant oligodendrocyte and neuronal apoptosis [153], suggesting that the glial inflammatory response to *Bb* could contribute to cell death. In addition, microarray analyses of tissue RNA revealed altered transcription of multiple genes that regulate the immune response as well as apoptosis [153]. When live *Bb* was inoculated into the CNS of rhesus macaques via the cisterna magna [154], within one week after inoculation there was a monocytic and lymphocytic pleocytosis and increased expression of IL-6, IL-8, CCL2, and CXCL13 in the CSF. Histopathological changes consistent with acute neurologic Lyme disease, showing leptomeningitis and radiculitis, as well as satellite glial cell and neuronal apoptosis in the DRG were also observed. IL-6 was produced by both astrocytes and neurons of spinal cord tissue and by neurons in the DRG. The chemokines CXCL13 and CCL2 were detected in microglia of the spinal cord. CCL2 was also detected in endothelial cells in the periventricular area of the brain. Other investigators have also confirmed the production of IL-6 and CXCL13 in *B. burgdorferi*-infected rhesus and human tissues [155–157].

Patients with chronic and recurrent neurologic Lyme disease who have persistent symptoms even after treatment are plagued primarily by pain, fatigue, and cognitive dysfunction. Elevated levels of IL-6 can cause symptoms of fatigue and malaise, common to many infectious and neurodegenerative diseases. IL-6 is pyrogenic, promotes B cell differentiation, stimulates the synthesis of acute phase reactants, and can also contribute to pain by increasing the sensitivity of nerve endings [158]. It is possible that IL-6 mediates the pain response in the sensory neurons of the DRG in LNB as well, since it was seen in DRG neurons of infected rhesus macaques [155, 158].

In a recent study, live *Bb* was shown to elicit the production of cytokines and chemokines, particularly IL-6,

IL-8, and CCL2, as well as to induce apoptosis in human oligodendrocytes cultured *in vitro* [159], by activating the enzyme caspase-3 [160]. Oligodendrocytes could therefore contribute to the elevated levels of cytokines and chemokines detected in the CSF of patients with LNB. Importantly, in the presence of the anti-inflammatory drug dexamethasone, a reduction in the amount of proinflammatory mediators, and a significant reduction in the *Bb*-induced oligodendrocyte apoptosis was observed [159]. This outcome is a strong indication that inflammation plays a role in mediating oligodendrocyte apoptosis, which could be mediated in part by the direct action of the spirochetes on oligodendrocytes or via inflammation mediated by *Bb* in oligodendrocytes.

Oligodendrocytes in brain tissue are especially vulnerable to demyelination as they are located immediately adjacent to the subarachnoid space, in the region known as the subpial space [161]. Oligodendrocytes are known to express receptors for various cytokines and chemokines [162]. Since inflammatory lesions are commonly found in the meninges in LNB, the myelitis that is seen in LNB may be in part due to oligodendrocyte dysfunction. These cells could be damaged by the inflammatory process initiated by the oligodendrocytes themselves, with participation of other glial cells, in addition to inflammatory mediators produced by the perivascular cellular infiltrates that are often present in CNS infection. As oligodendrocytes are vital for the survival and optimal function of neurons [162], oligodendrocyte damage could contribute to neuronal dysfunction and death and result in the impairment of CNS functions seen in patients with LNB. Caspase-mediated oligodendrocyte cell death has also been documented in inflammatory demyelinating diseases such as MS [163]. Cytokines and chemokines play a central role in inflammation, demyelination, and neurodegeneration in the CNS during inflammatory neurodegenerative diseases such as MS, which shows similar clinical signs as those shown by LNB [164].

The chemokine CCL2 reported in Lyme neuroborreliosis is of particular importance in mediating inflammation in neurodegenerative diseases [165, 166]. It is an important mediator in many neuroinflammatory and neurodegenerative brain diseases characterized by neuronal degeneration [167]. CCL2 has been found to be upregulated in actively demyelinating MS plaques [168], and its expression is increased in experimental autoimmune encephalomyelitis [169]. CCL2 modulates microglial activation and proliferation, thereby contributing to the inflammatory response mounted in the CNS [170]. Importantly, CCL2 levels are elevated in the CSF of patients with LNB [136], and high levels of CCL2 have been found in the CSF of rhesus monkeys infected intrathecally with *Bb* [154]. CCL2 is known to play a role in mediating nerve damage and demyelination of axons by causing an influx of monocytes and T cells in Wallerian degeneration [171] that may also possibly contribute to the axonal damage that affects patients with LNB of the PNS [172].

The cytokine IL-6 that has been reported in studies of LNB pathogenesis is known to be both helpful and harmful in the CNS [15, 173–176]. Dysregulated expression of IL-6 has been documented in several neurological disorders

such as MS, acute transverse myelitis, AD, schizophrenia, epileptic seizures, and PD [177]. In addition, IL-6 has been shown to be involved in multiple physiological CNS processes such as neuron homeostasis, astrogliogenesis, and neuronal differentiation [178]. IL-6 is known to promote oligodendrocyte and neuronal survival in the presence of glutamate-mediated excitotoxicity in hippocampal slices [179] and promotes survival of oligodendrocytes *in vitro* [180]. It is possible that IL-6 could mediate both neuroprotection as well as neurodegeneration in inflammatory neurodegenerative diseases including LNB.

The chemokine IL-8, also seen to be elevated in the CSF of LNB patients [132, 179] and in rhesus microglia, astrocytes, and endothelial cells exposed to *Bb* [151–154], is associated with BBB dysfunction and plays a central role in recruitment of neutrophils and T cells into the CNS during bacterial meningitis [181–183]. IL-8 is known to induce the expression of proinflammatory proteases, the matrix metalloproteinases MMP-2 and MMP-9, and proapoptotic protein Bim (Bcl-2-interacting mediator of cell death) and cell death, in cultured neurons in 24 hours [184]. There are reports indicating the presence of a cytolytic phenotype of IFN-γ-producing cells from patients with LNB [185, 186], suggesting the possible involvement of cytotoxic cells in mediating the demyelination and axonal degeneration seen in LNB [113, 116, 121].

B. burgdorferi has also been shown to induce the late production of significant quantities of the anti-inflammatory cytokine IL-10 in murine microglia and astrocytes [187]. The delayed production of IL-10 suggests that a possible negative feedback loop to limit potentially damaging inflammation within the brain parenchyma during persistent infections may be operating in parallel with the harmful effects of proinflammatory mediators. The treatment of primary rhesus macaque microglia with the tetracycline analogs doxycycline and minocycline resulted in attenuated microglial proinflammatory mediator responses to *Bb* [188]. Doxycycline is used for the treatment of Lyme disease patients and has been shown to improve adverse clinical symptoms at a time when viable spirochetes can no longer be easily detected. Therefore, the dampening of microglial proinflammatory cascades to limit unchecked neuroinflammation and subsequent neuronal damage may be beneficial to LNB patients.

4. Cytokines and Chemokines in Human Immunodeficiency Virus Encephalitis

Infection with the human immunodeficiency virus-1 (HIV-1) and acquired immunodeficiency syndrome (AIDS) are a persistent health problem worldwide. HIV-1 seems to enter the brain very soon after peripheral infection and can induce severe and debilitating neurological problems that include behavioral abnormalities, motor dysfunction, and dementia. The neurological manifestations directly related to HIV are acute viral meningitis, chronic meningitis, HIV-associated dementia (HAD), vacuolar myelopathy, and involvement of the peripheral nervous system [189]. Infected peripheral immune-competent cells, in particular macrophages,

appear to infiltrate the CNS and provoke a neuropathological response involving all cell types in the brain. HIV-1 encephalitis (HIVE), a common pathological manifestation of HAD, includes infiltration of macrophages into the brain where they become productively infected with the virus. This is accompanied by considerable cytokine and chemokine dysregulation in the brain that often culminates into the unique pathological features that characterize this syndrome. Once in the brain, HIV-1-infected blood-borne macrophages secrete proinflammatory cytokines such as TNF, IL-1β, and viral proteins such as HIV-1gp120 and Tat, which can affect neuronal function [190]. In the CNS, HIV-1 also incites activation of chemokine receptors, inflammatory mediators, extracellular matrix-degrading enzymes, and glutamate receptor-mediated excitotoxicity, all of which can initiate numerous downstream signaling pathways and disturb neuronal and glial function.

Lentiviruses are thought to enter the brain within circulating infected monocytes during immune surveillance. Numerous studies have been undertaken to determine the reasons underlying increased monocyte migration into the brain following lentiviral infection. HIV-infected leukocytes are primed for adhesion [191], having already shed L-selectin and increased expression of CD11b/CD18 compared with monocytes from healthy controls [192]. Therefore, it is possible that even marginal increases in the levels of chemokines expressed within the parenchyma would lead to increased migration of monocytes. Recent studies have shown that glial cells are stimulated to produce chemokines in response to inflammatory cytokines [193, 194] that are known to be secreted by simian immune-deficiency-virus- (SIV-)infected macrophages [195].

4.1. Astrocytes and Signaling in HIV Encephalitis.

Astrocytes act to repel circulating immune cells through secretion of eotaxin [196], reinforcing the brain's immune-privileged status in conjunction with the selective physical properties of the BBB. Under normal conditions the brain allows only limited access by immune cells. Early in HIV infection the virus enters the brain through normal trafficking. This leads to a transient increase in BBB permeability and a localized immune response. As the disease progresses to encephalitis, the immune response is dramatically increased, marked by a loss of tight junction integrity, gliosis, and formation of multinucleated giant cells in the parenchyma.

Astrocytes are the primary cell type found in glia scar formation [197, 198], and secrete cytokines and chemokines to elicit increased trafficking of leukocytes into the brain [193, 199, 200]. Astrocytes may also provide a role for the resolution of inflammation by reducing the secretion of proinflammatory cytokines, and increasing anti-inflammatory processes [198, 201, 202]. Decreased BBB integrity early in SIV/HIV infection allows latently infected monocytes to enter the brain [203]. Circulating virus could induce brain microvessel endothelial cells (BMEC) to express CD106 diffusely [204, 205] leading to increased monocyte migration into brain, where they become productively infected. Astrocytes respond to these macrophages resulting in a wide-range of cellular changes referred to as astrogliosis.

4.2. Astrogliosis.

On activation astrocytes undergo a morphological change, most notably an increase in ramification concomitant with upregulation of GFAP and thickened processes. Some astrocytes in the proximity of SIV lesions express peripherin, an alternative type III intermediate filament not normally expressed in brain [206]. Immunologically, astrocytes respond to HIV/SIV infection through increased production of inflammatory cytokines. As outlined above, the predominant inflammatory cell type in HIVE/SIVE is the monocyte-derived macrophage. The chemokines upregulated by astrocytes in HIVE/SIVE are largely specific to monocyte/macrophages [193, 207]. This suggests the possibility of a positive feedback system being initiated; a productively infected macrophage induces nearby astrocytes to up-regulate secretion of macrophage-specific chemokines, leading to lesion formation. The cytokine response of astrocytes includes a cornucopia of molecules including a variety of chemokines. It is intriguing that astrocytes will secrete a different "profile" of cytokines and chemokines in response to different classes of stimuli [208]. Below we discuss key cytokines and chemokines that are thought to play a role in SIVE/HIVE.

4.3. Microglia Activation and Cytokine Secretion.

Microglia serve as a "first responder" to neuroinvasion by pathogens. An actin binding protein, AIF-1, is considered to be a microglial-specific marker within normal brain [209–211]. As such, AIF-1 is ideally suited to examining morphological changes in microglia. Ramified microglia sample their environment using long processes. These processes retract on activation, allowing the microglia to migrate to the source of infection [212]. Cultured microglia also have a ramified morphology until activated by, for example, SIV-infected macrophages [213].

Surprisingly, the presence of macrophages is more important to the microglial response rather than whether they are infected with virus or not. IL-6 and IL-8 are both induced to be secreted by microglia when coincubated with macrophages, highlighting their role as "first responders" to infiltrating innate immune cells such as monocyte/macrophages.

4.4. Expression and Secretion of Selected Cytokines.

Productively infected macrophages in the encephalitic brain express TNF [195]. TNF-α receptors are present in the nonencephalitic brain [214], such that normal brains are primed to respond quickly to low levels of TNF. TNF induces increased chemokine production and secretion by astrocytes [215], and these chemokines induce monocyte migration preferentially over lymphocytes [193].

Vascular endothelial growth factor (VEGF) promotes proliferation of BMEC, resulting in reorganization of the cytoskeleton and tight junction proteins. This induces a decrease in BBB integrity, creating a permissive environment for monocyte migration, and also bidirectional leakage of proteins across the BBB. A possible mechanism for the VEGF pathway could be as follows: tat binds to the VEGF receptor [216], followed by the binding of the VEGF receptor to

focal adhesion kinase [217], increases of which have been implicated in BBB disruption [218].

Other proinflammatory cytokines, including IFN-γ and IL-6, are upregulated in the encephalitic brain, with far-reaching effects in neuroinflammatory events [219]. The complement pathway is also known to be induced through IFN-γ and IL-6 signaling, resulting in propagation of inflammation in the area surrounding lesions. There are well-characterized neurotoxicity manifestations associated with HIV infection [220], including increased secretion of the neurotoxic IL-6 by glia in response to gp120 [221]. Therefore, rapid secretion of high levels of IL-6 by microglia would be anticipated to be a detrimental effect of SIV-infected macrophage infiltration into the brain [222].

4.5. Expression and Secretion of Selected Chemokines. Levels of the chemokine IL-8 has been recently demonstrated to be elevated in microglia in HIVE brain tissue [223], possibly in response to gp120 [224]. IL-8 have been shown to have neurotoxic effects and thus, plays a role in cognitive dysfunction associated with HIV [225]. Increased IL-8 expression observed in glial nodules may be largely due to a factor secreted by HIV-infected macrophages [213].

An early study of chemokine expression in brains of macaques infected with SIV showed increased CCL3, CCL5, CCL7, and CXCL10 [207], although no increase in CCL2, CCL8 (MCP-2), or CXCL8 was observed in this study. Other later studies have produced conflicting results. Penton-Rol used dexamethasone to stimulate cells to have increased CCL2 receptors before infecting with HIV 89.6 [226]. The Clements group at Johns Hopkins has shown increased CCL2 mRNA in brain extracts using a highly accelerated encephalitis model [227], although mRNA does not always equate with secreted protein. Additionally, the Berman group at Einstein College of Medicine has shown numerous effects of CCL2 on HIV-infected macrophages [200, 228]. CCL2 was among several chemokines in CSF that were not upregulated in one study using humans infected with HIV [229], although IP-10 was upregulated. In contrast, CCL2 was increased in pigtail macaques that develop encephalitis [230]. The precise cell types producing these chemokines were not identified in these studies. CCL2 mRNA was upregulated in cultured astrocytes, but remained at low levels compared to CCL7, suggesting a role for CCL7 in HIV-related encephalitis [193].

Even under noninflamed conditions, CCL7 is expressed in the brain [193, 207], which could contribute to basal levels of monocyte migration into the brain for "routine surveillance" [231]. That CCL7 is upregulated by astrocytes in response to cytokines present in encephalitic brains gives a potential role for controlling monocyte migration during encephalitis as well [193, 207]. In more recent studies, stimulation of astrocytes with TNF induced an increase in secretion of numerous cytokines [215]. Analyses of gene arrays of astrocytes treated with TNF showed that the only cytokine upregulated was CCL7. It is also possible that astrocytes provide a role for the resolution of inflammation through reduction in secretion of proinflammatory cytokines and increasing anti-inflammatory processes [198, 201, 202]. In the above study, polygonal astrocytes stimulated with

TNF expressed higher levels of cytokines including VEGF than TNF-stimulated stellated astrocytes at the time points examined. Therefore, the order of stimulation of astrocytes is important in the subsequent secretion of cytokines [215].

5. Cytokines and Chemokines in Neuropathic Pain

Recent evidence suggests a strong correlation between inflammation following nerve damage and neuropathic pain [10, 23, 232]. Neuropathic pain is a complex syndrome resulting from many forms of peripheral nerve damage, for example, traumatic nerve injury, diabetes, infection, or drug-induced neuropathy, and immune and metabolic diseases [233]. Chronic pain can occur with peripheral nerve trauma and/or inflammation, autoimmune neuropathies and vasculitic neuropathies, or infection. Individuals who suffer from chronic pain experience prolonged pain at sites that may have been previously injured, yet are otherwise currently healthy. Chronic pain is associated with changes in neuroplasticity or changes in neural pathways, and synapses due to an erroneous reorganization of the nervous system, both peripherally and centrally. During the period of tissue damage, noxious stimuli and inflammation cause an elevation of nociceptive input from the periphery to the central nervous system. Prolonged nociception from the periphery elicits a neuroplastic response at the cortical level to change its somatotopic organization for the painful site, inducing central sensitization [234].

Peripheral nerves are the origin of almost all forms of neuropathic pain. Pain-responsive peripheral nerves reveal a remarkable degree of plasticity in both sensory neurons and spinal cord [10]. Immune processes can be directed against peripheral nerves, DRG, and dorsal roots resulting in pathological pain.

Immune activation near peripheral nerves may create increases in peripheral nerve excitability. Infectious agents as well as proinflammatory mediators produced by activated microglia can cause alterations in the blood-nerve barrier (BNB) as a result of chemoattractant molecules released at the site of the damaged peripheral nerve, which in turn recruit neutrophils and macrophages from the circulation into the nerve. Proinflammatory cytokines participate in this immune activation and orchestrate the early immune response. However, these inflammatory mediators can directly increase nerve excitability, damage myelin, and alter the permeability of the BNB leading to edema and further infiltration of immune cells. Schwann cells that ensheath peripheral nerves are macrophage-like and can present nonself-substances to T lymphocytes to further activate the immune cells. Schwann cells also participate in the removal of damaged myelin and cellular debris. Importantly, Schwann cells rapidly release the chemo-attractant CCL2 upon nerve damage that in turn recruits monocytes and T cells to the site of the nerve degeneration [10]. Proinflammatory cytokines have been repeatedly implicated in demyelination and degeneration of peripheral nerves, increases in sensory afferent excitability, and induction of neuropathic pain [23].

Inflammatory mediators elicited in the cells of the DRG and those produced by infiltrating immune cells and spinal microglial activation are key elements that mediate the signal transduction of the pain response [235]. Macrophages, lymphocytes, and satellite glial cells in the DRG and in the dorsal horn of the spinal cord participate in neuroimmune activation of glial cells, promoting the development of neuropathic pain. Since some chemokine receptors such as CCR2, CCR5, CXCR4, and CX3CR1 are located in primary afferent neurons or secondary neurons of the spinal dorsal horn [236], their chemokine ligands can potentially alter pain transmission. Peripheral administration of the chemokines CCL2, CCL3, CCL5, and CXCL12 has been shown to produce pain behaviors that are elicited by the activation of chemokine receptors in the DRG [236].

Importantly, CCL2 participates in pain regulation by directly interacting with sensory neurons and indirectly via peripheral leukocyte activation in the PNS [236, 237]. CCL2 has been shown to be elevated in primary sensory neurons after nerve injury, and CCR2 expression has been observed in both DRG neurons and activated Schwann cells in injured peripheral nerves [237]. Moreover, neuropathic pain induced by nerve injury is not elicited in CCR2 gene-deficient mice [236]. The addition of CCL2 to cultured DRG neurons triggered the release of calcitonin gene-related peptide (CGRP), a nociceptor neurotransmitter, from these cells, presumably as a result of increased neuronal excitation [238].

CCL3 has been found to be upregulated in activated Schwann cells and in infiltrating macrophages close to injured nerves. This chemokine participates in the development of neuropathic pain through its receptors CCR1 and CCR5, which are located in Schwann cells and macrophages [239]. Interestingly, there is an increase of fractalkine (CX3CL1), which is known to be both a pro- and anti-inflammatory molecule in injured nerves, and localization of its receptor CX3CR1 in recruited macrophages and DRG neurons. The activation of fractalkine-CX3CR1 has been shown to attenuate peripheral nerve injury-induced neuropathic pain [240].

The crosstalk between glial cells and neurons is important in the development of neuropathic pain [234]. Proinflammatory cytokines such as IL-1β, IL-6, and TNF produced by glial cells and neurons accelerate central pain sensitization, and inhibition of these cytokines in the CNS and PNS effectively reduces neuropathic pain [241]. Brain-derived neurotrophic factor (BDNF) derived from activated microglia potentiates the excitability of spinal neurons [242]. Microglial IL-18, a member of the IL-1 family, also plays a pivotal role in neuropathic pain [243]. IL-1β produced by macrophages and Schwann cells in injured nerves directly sensitizes nociceptors in primary afferent neurons [244]. IL-1 induces the release of substance P from DRG neurons [245] and neuropathic pain is reduced in IL-6 KO mice [246]. IL-6 can also contribute to pain by increasing the sensitivity of nerve endings [246]. IL-6 can enhance neuropathic pain in the dorsal horn by activating STAT3 signaling in glial cells after peripheral nerve injury. The STAT-3 pathway is a key mediator of signal transduction in neuropathic pain [247].

A recent study evaluated the role of axonal transport in neuroimmune communication following peripheral nerve injury, linking focal changes in Schwann cell activation and release of the proinflammatory cytokine TNF with subsequent activation and sensitization of ascending sensory neurons and glia that culminate in the neuropathic pain state. New data demonstrate that axonally transported (biotinylated) TNF-α activates and localizes with dorsal horn astrocytes within 96 hours after injection into the sciatic nerve and that glial GFAP-activation in these glial cells is diminished in TNF-α-receptor 1 KO mice [248].

IL-17 is an important regulator of immune responses and is involved in inducing and mediating proinflammatory reactions in a wide range of inflammatory and autoimmune diseases of the nervous system. Using IL-17 KO mice, it has been demonstrated that IL-17 contributes to neuroinflammatory responses and pain hypersensitivity following neuropathic injury [249]. Compared to wild-type, IL-17 KO mice displayed significantly decreased mechanical pain hypersensitivity as well as decreased infiltration of T cells and macrophages to the injured sciatic nerves, and the L3-L5 DRG, and decreased activation of microglia and astrocytes in the L3-L5 dorsal and ventral horns of the spinal cord. This work shows that IL-17 contributes to neuroinflammation and neuropathic pain following peripheral nerve injury and identifies IL-17 as a potential therapeutic target for treating neuropathic pain.

Recent studies have suggested that the C-C chemokine receptor (CCR)5 interacts with μ-opioid receptor and modifies a nociceptive reaction [250]. A study that examined effects of CCR5 deficiency on pain responses by employing CCR5 KO mice found that pain responses of CCR5 KO mice to chemical or inflammatory stimuli were milder than those of CCR5 wild-type mice [251]. Though the roles of proinflammatory cytokines and chemokines in neuropathic pain have been identified [252], the precise relationship between the chemokine-cytokine network and neuropathic pain is not yet well understood. Further studies are needed to understand the neuropathic regulatory mechanisms underlying neuroinflammation after nerve injury.

6. Conclusion

Cytokines and chemokines play an important role in mediating neuroinflammation and neurodegeneration in various kinds of inflammatory neurodegenerative diseases including bacterial meningitis, brain abscesses, Lyme neuroborreliosis, and HIV encephalitis described above. Interestingly, recent evidence suggests that peripheral and central neuroinflammation associated with cytokine-chemokine networks following nerve damage also play a central role in the pathogenesis of neuropathic pain. Although a link has been established between cytokines, chemokines, and neurodegeneration, their signaling mechanisms are complex and appear to involve a balance between promoting cell survival, apoptosis, and proinflammatory responses.

Although inflammation may induce beneficial effects such as pathogen clearance and phagocytosis of debris and apoptotic cells besides tissue repair processes, uncontrolled inflammation can result in detrimental outcomes via the production of neurotoxic factors that exacerbate

neurodegenerative pathology. The factors that may disrupt this normal equilibrium remain largely unknown. Several cytokines and chemokines and their receptors orchestrate this immune response. Further, anti-inflammatory responses are regulated by proteins that inhibit signal transduction pathways, such as suppressor of cytokine signaling proteins, transcriptional repressors, and anti-inflammatory molecules that help control excessive inflammation. Chemokine recruitment of inflammatory cells to the sites of injury is instrumental in driving a secondary damage cascade.

Given the mounting evidence for their role in neurodegenerative disorders, cytokines and chemokines have received considerable attention as therapeutic targets. Considering that inflammation mediated by cytokines and chemokines is a common denominator in neurodegenerative diseases, targeting the correct timing of an immune response will be a pivotal factor in designing successful therapies. Further, it will be a challenge to design therapeutic agents that safely and effectively target only the detrimental mechanisms that contribute to disease pathogenesis, as cytokines and chemokines are vital for the normal functioning of the body. An understanding of the factors that dictate the switch from a protective to a deleterious inflammatory response will make possible interventions able to limit tissue damage. This type of intervention will also require a thorough understanding of the cell-subtype-specific action and cellular signaling pathways in CNS and PNS injury in order to design selective drug targets. Further investigations aimed at understanding chemokine-cytokine networks that are operative in the signal transduction of the pain response will prove beneficial in designing novel therapeutic strategies to alleviate neuropathic pain, a significant factor in neurodegenerative diseases.

Acknowledgments

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Review Article

MicroRNAs as Novel Regulators of Neuroinflammation

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MicroRNAs are relatively recently discovered class of small noncoding RNAs, which function as important regulators of gene expression. They fine-tune protein expression either by translational inhibition or mRNA degradation. MicroRNAs act as regulators of diverse cellular processes, such as cell differentiation, proliferation, and apoptosis. Their defective biogenesis or function has been identified in various pathological conditions, like inflammation, neurodegeneration, or autoimmunity. Multiple sclerosis is one of the predominated debilitating neurological diseases affecting mainly young adults. It is a multifactorial disorder of as yet unknown aetiology. As far, it is suggested that interplay between genetic and environmental factors is responsible for MS pathogenesis. The role of microRNAs in this pathology is now extensively studied. Here, we want to review the current knowledge of microRNAs role in multiple sclerosis.

1. Introduction

For a long time, the brain was considered as an immune privilege organ. This phenomenon was defined as a complete inaccessibility for the immune cells and immune mediators, mainly due to the impermeability of the blood-brain barrier (BBB) [1]. In the light of relatively recently obtained results from multiple studies, brain "immune privilege" has to be redefined. Now, it is considered that this term is mainly related to the specific BBB architecture, brain-resident cells immunoregulatory function, and their microenvironment, which results in restricted access of immune system elements to the central nervous system (CNS) [2, 3].

It has been proposed that specific morphological architecture of CNS borders is crucial for maintaining its immune privilege. The BBB and the blood-cerebrospinal fluid barrier (BCSFB), as an outer element of CNS borders, may be breached by activated immune cells. After migration through the brain barriers, immune cells target the cerebrospinal fluid-drained leptomeningeal and perivascular spaces [4]. The inner elements of the CNS border are glia limitans, built of astrocytic foot processes and parenchymal basement membrane [5]. Within the CSF-drained leptomeningeal and perivascular spaces, macrophages are present, which can act as antigen presenting cells (APCs) for the activated T cells [6]. After recognizing specific antigens, T cells become reactivated and result in accumulation of additional immune cells. In this stage, the inner barrier may be disturbed, and immune cells and various mediators act inside the brain [7]. Thus, in physiological conditions, CNS homeostasis is ensured by permission for immune cells migration through the BBB and BCSFB only to the CSF space, where in the absence of antigens, they patrol CNS barriers.

Other features related to brain immune privilege include absence of the lymphatic vessels in the parenchyma, which allow in other organs for draining antibodies and immune cells to peripheral lymph nodes, low expression of MHC class II on CNS resident cells, and deficiency of dendritic cells (DCs) in the parenchyma [8–10]. The immune privilege of the brain is also connected with specific CNS-driven mechanisms regulating T cells functions within CNS. Brain resident cells, namely, neurons and glia, may actively regulate macrophage and lymphocyte responses [11, 12]. It is important to notice that immune privilege is not applied for all brain regions. This phenomenon is restricted mainly

to the parenchyma proper. Other regions of CNS, the ventricles, meninges and subarachnoid spaces, demonstrate immune reactivity similar to that seen on the periphery [13].

In pathological conditions, such immune privilege is disrupted leading to the development of inflammation and/or neurodegeneration, which are hallmarks of various CNS diseases, for example, Alzheimer's disease, Parkinson's disease, and multiple sclerosis (MS).

1.1. Neuroinflammation in Multiple Sclerosis. Multiple sclerosis is a chronic inflammatory, neurodegenerative disorder characterized by CNS infiltration of autoreactive immune cells, demyelination, acute astrogliosis, and axonal loss. The aetiology of MS is still not known, but it is widely appreciated that the disease is a result of complex interplay between genetic and environmental factors [14, 15]. Progression of this disorder leads to many neurological dysfunctions, such as loss of vision, loss of sensation, and problems with walking. About 80% of MS patients develop relapsing-remitting form of disease, while 10–15% presents primary progressive form. However, after about 10 years, roughly half of relapsing-remitting patients develop a secondary progressive stage of disease [16]. The presence of various forms of disease and differential immunopathology points toward the important role of various subsets of T-helper cells and their relative proportion present at the site of inflammation [17].

It was considered for a long period of time that T-helper type 1 (Th1) cells were the major effectors in MS pathophysiology. Th1 cells are characterized by the expression of the transcription factor T-bet and the IFN-γ production [18]. However, more recently, a new subset of T-helper cells have been identified, namely, Th17 cells. This subpopulation is characterized by expression of the retinoic acid receptor-related orphan receptor alpha and gamma t (ROR-α and ROR-γt) and by the production of IL-17 [19]. It was reported that Th17 cells better attach to brain endothelium than Th1 cells, in part due to the presence of CD146 on their surface [20], and they are more effective in migration through the BBB, as they express high levels of CCR6 and CD6 [21]. Moreover, it was shown that IL-17 leads to the BBB breakdown. This cytokine is also a potent inducer of neutrophil infiltration to the site of inflammation [22]. Recruited neutrophils activate various enzymes such as matrix metalloproteinases (MMPs), proteases, and gelatinases participating in further BBB disruption [23, 24]. Studies conducted on experimental autoimmune encephalomyelitis (EAE), an animal model of MS, shows, however, that Th17 cells are not sufficient for disease induction. These results suggest that Th17 subset together with Th1 cells is responsible for disease development [25].

Another important subpopulation of CD4 + T cells—T-helper type 2 (Th2) cells—is also important for MS pathology, as it was reported that their response results in disease amelioration [26]. Regulatory T cells (Tregs) also fulfilled protective function, which has been manifested in the control of autoimmune diseases and prevention of their progression. However, in multiple sclerosis, the function but not their frequency is impaired, leading to disease progression [27].

CD8 + T cells are also implicated in MS pathology, as the clonal and oligoclonal expansion of myelin antigen-reactive CD8 + subset was observed within MS plaques [28].

Activated T cells express on their surface high levels of molecules, like very late antigen-4 (VLA-4) and leukocyte-function-associated antigen-1 (LFA-1), which has improved their adhesion to the brain endothelium and subsequent migration across BBB [29–31]. After such migration T cells undergo antigen restimulation, resulting in their accumulation and proliferation. Reactivated T cells release proinflammatory molecules, which CNS resident cells, macrophages, and B cells [32, 33]. B cells and plasma cells contribute to MS pathology, as they were detected in brain and CSF of MS patients. What is more, antibodies directed against myelin antigens have been reported in the serum of MS patients [34–36].

Microglia are the resident macrophages of the CNS. In physiological conditions, they display a quiescent phenotype that is characterized by a CD45 phenotype and lowered expression of MHC class II, B 7.2, and CD40 [37]. In stress condition they undergo morphological changes, develop phagocytic abilities, and upregulate MHC class II, B7.2, and CD40 expression becoming highly activated [37–39]. Microglia play important role in response to pathological stimuli affecting CNS, as it was shown that the overproduction of their secreted factors, such as TNF-α, contributed to the development and progression of MS [40].

Astrocytes, together with microglia cells, participate in innate inflammatory responses in CNS. Astrocytes react to pathogen/danger signals by cytoskeletal rearrangements associated with an increase in glial fibrillary acidic protein (GFAP) and process extension, which are the hallmark of a reactive astrogliosis, process seen in MS patients [41, 42]. They secrete interferons, thought to be crucial in the CNS defense mechanism against diverse inflammatory factors. However, prolonged unopposed proinflammatory cytokine signaling could have harmful consequences leading to pathological inflammation and neurodegeneration. Recruitment of MyD88 to the toll-IL-1 receptor (TIR) domain of the IL-1 receptor is essential in the cell signaling pathways underlying astrocyte-mediated inflammation and neurotoxicity [41, 42]. Macrophages are the major MHC class II positive cells. They have integral role in disease initiation in EAE. However, in MS pathology, they are not the only class II positive cells as the monocytes, DCs, microglia, and astrocytes could also act as an antigen presenting cells [43].

Members of the toll-like receptor (TLR) family are thought to be the primary evolutionarily conserved sensors of pathogen-associated molecular patterns [44]. Binding of the appropriate ligand to TLRs initiates molecular cascade leading to phagocytosis, production of a variety of cytokines, and subsequently regulation of inflammatory reaction and adaptive immune response [45]. In neuroinflammation, TLR activation may modulate the production of inflammatory cytokines [46]. The increase in TLRs expression was observed in MS brain lesions, CSF mononuclear cells, and also EAE [47, 48].

1.2. Biogenesis and Function of MicroRNAs. MicroRNA (miR) is a relatively novel class of small noncoding RNA, demonstrating regulatory function to mRNA translation. MiRs are approximately 22 nt long single-stranded molecules, encoded in intergenic regions, introns, exons, exon overlaps, or UTR regions [49]. They may be present as single genes, or they are arranged in clusters [50]. MiRs may be expressed as independent genes with their own transcriptional regulatory elements or from intronic sequences of protein-coding genes [50]. The presence of miR clusters may be evidence of their structural or functional (targeting mRNAs of proteins involved in the same cellular pathway) similarity between encoded miRs [51]. Most of microRNAs are transcribed by the RNA polymerase II [52], whereas some of them are results of RNA polymerase III activity [53]. They are usually transcribed as a primary transcript (pri-miRNA), which is usually several kilobases long, and contain stem-loop structures [52]. Pri-miRNA is processed in the nucleus by the microprocessor complex composed of a processing enzyme Drosha and RNA binding protein, DGCR8/Pasha [54]. This enzymatic complex performs asymmetric cleavage which generate about 70 nt long pre-miRNA containing a two nt $3'$ overhang [55], essential for nuclear export [56, 57]. Pre-miRNA is transported to the cytoplasm by exportin 5 and Ran GTPase for final processing by the RNAse III enzyme Dicer, specialized to bind RNA ends, especially with short $3'$ overhangs. Dicer release an approximately 22 nt double-stranded miR with a $5'$ phosphate end [58]. Next, duplex RNA is incorporated into a protein complex named RNA-induced silencing complex (RISC), unwound by a helicase and separated to two ssRNAs [59]. The key protein players of RISC are RNA binding protein Argonaute (Ago) and its RNA binding partner, TRBP. The guide strand is thermodynamically favored for incorporation to the Ago complex as it has a less stable $5'$ end than passenger strand, which mostly undergoes degradation [55].

MicroRNAs fine-tune the production of proteins within cells through repression or activation of mRNA translation [60]. They act through the interaction of their seed region mainly with the $3'$ untranslated region (UTR) of the given mRNA, as it was recently shown that they can interact also with $5'$ UTR or protein coding region [61, 62]. Mature miR altered mRNA expression by either inhibiting translation or signaling for mRNA degradation, depending on the degree of sequence complementarity between seed region located on the $5'$ end of miR (between 2 and 8 nt) and binding site of mRNA, although sequences outside the seed region are also important for recognizing targets and optimizing mRNA regulation [63]. The seed area may be supplemented by nucleotide 8 of miR, by adenine from nucleotide 1 of miR, or by both of them. The newly discovered microRNA's seed region comprises of nucleotides 3 to 8 [64–66].

MiRs are universal regulators of protein expression, as a single molecule can regulate translation of hundreds of targeted mRNAs and single mRNA's $3'$ UTR may have multiple binding sites for various microRNAs. MiRs may function in two ways to enhance their regulatory capacity, by targeting multiple binding sites present within $3'$ UTR of mRNA or by targeting multiple genes from the same cellular pathway [67]. It is estimated that in mammals, miRs may regulate more than 60% of protein-coding genes [67]. Moreover, microRNAs may function not only in cytoplasm, as they were also identified in the nucleus [68, 69], where they may act as an epigenetic regulators of gene expression [70].

MicroRNAs play crucial role in the regulation of diverse biological processes, like tissue development and homeostasis [71], cell proliferation and differentiation, apoptosis, and immune system function [72]. They are crucial for system's ability to coping with external and internal perturbations, as they regulate the mRNA expression profile by reinforcing transcription, reducing defective and overabundant transcript copy number [67]. Altered biogenesis and/or function of miR is implicated in the various pathological processes such as autoimmunity, viral infections, neurodegeneration, and inflammation [73]. Dysregulated miRs contribute to the development of various diseases, for example, cancer, cardiovascular, or neurological diseases [71, 74, 75]. It was shown that inflammation may regulate miR biogenesis. TLR ligands, antigens, or cytokines can alter miR expression level through specific transcription factors regulation [76–78]. It was also reported that cytokines may lead to deregulation of Dicer expression resulting in aberrant pre-microRNA processing [79].

Defective miR regulation during diverse immune processes may be associated with several human diseases. There are various processes, except for the impact of inflammatory factors, contributing to such regulation such as mutations, epigenetic inactivation, or gene amplification [80].

1.3. The Role of miRs in Neuroinflammation and MS. In the light of rapidly accumulating data from various studies, it has been concluded that miRNAs are crucial regulators of immune cell development and function. Diverse alterations in their biogenesis and regulatory role have been observed in inflammatory diseases such as rheumatoid arthritis, psoriasis, and multiple sclerosis. As multiple sclerosis is one of the most common neurological debilitating disease of as yet unknown etiology, we want to review in this section current knowledge regarding the role of these small noncoding RNAs in the MS inflammation (Table 1).

Multiple sclerosis is considered as a T-cell-mediated disorder, so it is not surprising that researchers attention is directed toward the role of miRs deregulation in T-cell maturation, activation, and function. One of the first identified miRs related to the T cells is miR-155. Expression of this miR has been linked to T cells activation following TCR stimulation [81, 82]. Differentiation of T-helper cells is also dependent on miR-155 expression. Mice deficient in this miR have demonstrated normal lymphocyte development, but altered Th1/Th2 ratio with presence of increased Th2 polarization and elevated levels of Th2 cytokine production [83–85]. Studies conducted by Cox et al. on MS patients identified significant downregulation of hsa-miR-17 and hsa-miR-20a [86]. Using knock-in and knock-down approaches it was concluded that these two miRs participate in T-cell activation regulation. FOXO1, belonging to forkhead family transcription factors, is a suppressor of T-cell proliferation, activation, and differentiation. Downregulation of FOXO1

TABLE 1: MicroRNA regulation of inflammatory cells differentiation and function.

Cell type	Process	MicroRNA	Notes
T cells	T-cell differentiation	miR-155	—
		miR-182-5p	Regulation of FOXO1 expression
		miR-146	High level in Th1, low level in Th2, and regulation of IL-17A expression
		miR-21	Regulation of Th1 differentiation and IFNγ secretion, positive regulator of Foxp3 expression
		miR-326	Th17 differentiation through regulation of Ets-1 expression
		miR-301a	Th17 differentiation through regulation of PIAS3 expression, regulation of IL-17 secretion, and RORα and RORγt expression
		miR-31	Negative regulator of Foxp3 expression
	T-cell activation	miR-155	—
		miR-17	—
		miR-20a	—
		miR-182-5p	Regulation of FOXO1 expression
		miR-301a	CD8+ activation through CD69 regulation
		miR-146	Regulation of Treg function
		miR-17-92	Regulation of Treg function
		miR-142-3p	Regulation of Treg function
	Sensitivity to Ag	miR-181a	Regulation by targeting, for example, SHP-2, DUSP5, and DUSP6
B cells	Pro-B to pre-B stage transition	miR-181a	—
		miR-17-92	Antagonist of proapoptotic genes
		miR-150	Regulation of c-Myb expression
	B-cell differentiation	miR-181a	Positive regulator
	Response to Ag/Ig production	miR-155	Regulation of response to various antigens, Ig class switching to IgG, Ig gene diversification, and extrafollicular and germinal center responses
		miR-181b	Regulation of Ig class switch recombination
Granulocytes	Granulocytopoiesis	miR-223	Regulation of Mef2c expression
Microglia	Quiescent phenotype	miR-124	Regulation of CEBPα/PU.1 pathway
	Inflammatory response	miR-155	Regulation of SOCS-1 expression
Astrocytes	Inflammatory response	miR-146a	Negative feedback regulator
		miR-155	Regulation of proinflammatory gene expression
Monocytes	Monocytopoiesis	miR-17-5p	Regulation of AML1 expression
		miR-20a	Regulation of AML1 expression
		miR-106a	Regulation of AML1 expression
	Monocyte differentiation	miR-424	—
Macrophages	Macrophage activation	miR-155	Regulation of CD47 expression
		miR-326	Regulation of CD47 expression
		miR-34a	Regulation of CD47 expression
Dendritic cells	APC function	miR-155	—
	DC differentiation	miR-34	Regulation of Jagged1 and WNT1 expression
		miR-21	Regulation of Jagged1 and WNT1 expression
Endothelial cells	Cell migration	miR-17	Regulation of ICAM1 expression
		miR-126	Regulation of VCAM1 expression

expression, in part by hsa-miR-182-5p, is crucial for the T-cell clonal expansion [87].

It has been suggested that miR-146a expression may play a role in cell fate determination. Studies conducted on mouse lymphocytes have shown that the level of miR-146a is increased in Th1 cells and decreased in Th2 cells, when compared to its expression in naive T cells [88]. The polarization of Th1 cells may be in part regulated also by miR-21, as IL-12p35 is one of its potential targets. IL-12p35 is a subunit of IL-12 [89], cytokine which controls Th1 differentiation and IFN-γ secretion by the synergistic action with IL-18 [90].

Du et al. indicated, in the studies conducted on MS Chinese patients, that miR-326 is a regulator of Th17 cells differentiation [91]. It was shown that *in vivo* silencing of miR-326 caused reduced number of Th17 subset and mild EAE, whereas its overexpression resulted in elevated level of Th17 cells and more severe EAE. It was concluded that miR-326 acts on Ets-1, a negative regulator of Th17 differentiation [91]. Mycko et al. reported significant upregulation of another miR, namely, miR-301a in T-helper cells in response to MOG antigen [92]. MiR-301a regulates Th17 differentiation through inhibition of PIAS3, a negative regulator of the STAT3 activation pathway [92]. Inhibition of miR-301a results also in decreased secretion of IL-17 and downregulation of ROR-α and ROR-γt expression [92]. Moreover, IL-17A expression may be inhibited by miR-146 function [93]. O'Connell et al. have revealed in MS animal model the positive role of miR-155 in autoimmunity as this miR drives Th17 differentiation of T cells [94]. As mentioned earlier, miR-301a regulates Th17 differentiation. However, it was reported that this microRNA is also expressed due to CD8 + T cells activation, where it may function as a regulator of CD69 expression [95].

MicroRNAs play important roles in regulatory T cells (Tregs) that are important protective cells preventing development and progression of autoimmune diseases. MiR-155 was shown to regulate Treg development, as miR-155-deficient mice have reduced numbers of Tregs [96], whereas miR-146 and miR-17-92 cluster regulate Treg function [97]. MiR-146a, when highly expressed in this T cell subset, selectively controls Treg-mediated inhibition of IFN-γ-dependent Th1 response and inflammation by activating STAT1 expression [98]. It was also reported that in human Tregs miR-21 functions as a positive indirect regulator of Foxp3 expression, while miR-31 acts as its negative regulator [99]. Recently, it was shown that Foxp3 represses miR-142-3p expression, leading to exacerbation in cAMP production and suppressor function of Treg cells [100].

Development of bone marrow-derived B cells is partially regulated by miR-181a expression. During B-cell development from the pro-B to the pre-B-cell stage, the expression level of miR-181a decreases [101]. Upregulated expression of miR-181a in pro-B stage inhibits such stage transition. MiR-181a is also considered as a positive regulator of B cells differentiation, as its expression in hematopoietic stem and progenitor cells leads to an increase in fraction of B-lineage cells and decrease in T cells or myeloid cells [101]. Conditional deletion of Dicer in mouse B cells also results in complete B cell development

blockage [102]. Similar results were obtained for miR-17-92-deficient B-cells. Inhibition of miR-17-92 expression results in elevated levels of proapoptotic protein Bim and inhibition of B cell development at the pro-B to pre-B stage [103]. MiR-150 is known for its role in B lymphocytes development. It was shown that its constitutive expression may lead to similar results as seen for Dicer- and miR-17-92-deficient mouse [104]. MiR-150 controls B-cell differentiation by targeting transcription factor—c-Myb [105].

As observed for the first time in T cells, miR-155 is crucial also for B-cell functions. It has been reported that miR-155 is important in B-cell responses to thymus-dependent and- independent antigens [85]. It was also shown that miR-155 regulates immunoglobulin class switching to IgG [83]. Elevated expression of PU.1, a target for miR-155, leads to the reduced production of IgG1 cells. This suggests that miR-155 regulation of PU.1 may be in part responsible for proper generation of immunoglobulin class-switched plasma cells [85]. MiR-155 also represses activation-induced cytidine deaminase, enzyme essential for immunoglobulin gene diversification [106, 107]. Moreover, miR-155-deficient B cells generated reduced extrafollicular and germinal center responses [85]. Recently, immunoglobulin class switch recombination was also connected with the function of miR-181b. Elevated expression of miR-181b results in impairment of this process [108].

MiR-223 is mainly expressed in myeloid cells and functions as a regulator of granulocytopoiesis. It was reported that miR-223 negatively regulates both the proliferation and activation of neutrophils by targeting Mef2c, a transcription factor promoting myeloid progenitor proliferation [109]. Moreover, neutrophils deficient in this miR are hypermature and hypersensitive to activating stimuli and that they display aberrant pattern of lineage-specific marker expression [109]. However, there are contradictory results from different study indicating that miR-223 is a positive regulator of granulocytopoiesis [110]. Additionally, miR-223 modulates the NF-κB pathway leading to alterations in immune inflammatory responses [111]. This opposed results may reflect complex interplay between the miRNA and its target pathway. It was reported that another miR, namely, miR-9, is similarly upregulated in human peripheral monocytes and neutrophils. This upregulation is mediated by proinflammatory signals conveyed in a MyD88- and NF-κB-dependent manner [112].

Results obtained from numerous studies have shown that expression of toll-like receptors (TLRs) may be regulated by miR-146a. Expression of miR-146a was significantly upregulated by TNF-α and IL-1β and blocked by its receptor antagonist. Interestingly, miR-146a acts through suppression of proinflammatory proteins such as interleukin-1 receptor-associated kinase 1/2 (IRAK1/2) and TNF receptor-associated factor (TRAF) as well as IL-1β in a negative feedback loop [113]. It may also directly interacts with complement factor H (CFH), a repressor of the inflammatory reaction, leading to exacerbation of inflammation [114, 115].

Ponomarev et al. provided evidence that miR-124 has crucial role in maintaining quiescent phenotype of microglia

in mouse EAE—experimental model of MS [116]. Expression of miR-124 was significantly downregulated in activated microglia, resulting in subsequent upregulation of CCAAT enhancer-binding proteins (C/EBPalpha) and PU.1 expression. PU.1 plays important role in the activation of monocytic lineage phenotype [117]. During EAE, expression of brain-specific miR-124 was observed only in microglia, suggesting that this small noncoding RNA participates in the resting phenotype of these cells through the regulation of C/EBPalpha/PU.1 pathway [116]. It was shown that immune response in microglia could be modulated by miR-155. MiR-155 decreases expression level of suppressor of cytokine signaling 1 (SOCS-1) leading to elevated cytokine and NO production [118]. Recently, studies conducted by Iyer et al. reported regulatory role of miR-146a in astrocyte-mediated inflammatory response [113]. In addition, it was reported that in multiple sclerosis lesions miR-155 is highly expressed in reactive astrocytes [119]. By the application of miR-155 inhibitor oligonucleotide, Tarassishin et al. have shown that miR-155 regulates astrocyte proinflammatory gene expression [120].

It was reported by Fontana et al. that monocytopoiesis is partially controlled by three miRNAs: miR-17-5p, miR-20a, and miR-106a. These microRNAs regulate expression of transcription factor acute myeloid leukaemia-1 (AML1) [121]. However, AML1 binds to and transcriptionally inhibits expression of those three miRs in a negative feedback loop [121]. Another transcription factor related to monocyte differentiation, PU.1, activates transcription of miR-424. Upregulation of miR-424 stimulates monocyte differentiation [122]. Studies by Junker et al. conducted in active MS lesions identified three upregulated miRNAs: miR-155, miR-326, and miR-34a that target the same transcript—CD47 mRNA [119]. CD47 is a membrane glycoprotein, which mediates macrophage inhibition. The interaction of CD47 with signal regulatory protein-α present on macrophages inhibits IgG or complement-induced phagocytosis. Downregulation of CD47 expression results in promotion of myelin phagocytosis by macrophages during MS course [123, 124].

The regulation of miRs is seen also in dendritic cells (DCs). Deficiency in miR-155 was shown to affect their function as an APC in EAE [83]. It was also reported that miR-155 knockdown results in increase in the proinflammatory cytokine IL-1β expression [125]. Other miRs related to DCs are miR-34 and miR-21. They were reported to play important role in myeloid-derived DC differentiation through regulation of Jagged1 and WNT1 mRNA translation [126].

The induction of central tolerance is regulated during T-cell maturation to maintain proper immune system functioning. There is evidence for strong correlation between the sensitivity of the T cells to antigen and levels of miR-181a [127]. A decrease in TCR sensitivity may result in self-tolerance breakdown and subsequent autoimmunity development [128]. The high levels of miR-181a may contribute to the decreased activation threshold of autoreactive T cells, while inhibition of miR-181a expression in the immature T cells lowers their sensitivity. The function of miR-181a is mainly mediated by downregulation of several protein tyrosine phosphatases, such as SHP-2, DUSP5, and DUSP6 [129].

The process of immune cells recruitment into the brain parenchyma is also regulated by microRNAs. It was revealed that miR-17 and miR-126 targeted ICAM1 and VCAM1 mRNA, respectively [130, 131]. Moreover, it was shown that miR-124 and -126 have regulated expression of CCL2, a chemokine responsible for monocytes recruitment to brain parenchyma. Hence, miRNAs associated with inflammatory response may also act as a potential neuroprotectants [132, 133].

2. Conclusions

Inflammation is an extremely important and complex biological process of the immune system activated in response to harmful stimuli such as diverse pathogens or cell damage. Its main physiological function is manifested in removal of pathogens and damaged cells or healing process [134]. However, in some circumstances, inflammatory response may be unleashed from the biological control leading to tissue damage. Dysregulated inflammatory reaction can result in development of autoimmune disorders such as rheumatoid arthritis, psoriasis, or multiple sclerosis [135, 136].

Multiple sclerosis is a multifactorial neurological disease characterized by the presence of inflammatory brain infiltrates and subsequent neurodegeneration. MS is a progressive disorder affecting mostly young adults. It is stated that MS develops in genetic susceptibility individuals, which are exposed for action of various predisposing environmental factors. Although multiple sclerosis has been studied for many years, exact factors underlying its pathogenesis remain still unknown.

It has been recently shown that less than 2% of human genome undergoes translation into proteins. However, more than half of the human genome is transcribed, suggesting that most of the transcripts account for noncoding RNAs (ncRNAs). It has now become obvious that such RNA molecules are not the "junk sequences" as it was thought before. Rather, they demonstrate important regulatory role [137]. Noncoding RNAs may be divided into two groups: long and short ncRNAs. Within each of these groups, we can further distinguish various subtypes. Most of them have not known or only partially discovered function. One of the most extensively studied groups of ncRNAs are microRNAs. These small RNAs are crucial posttranscriptional regulators altering diverse cellular processes. It was reported that they are important fine-tuners of immune responses. Both the induction and repression of miRNA expression mediated by various inflammatory stimuli may lead to alteration in immune cells differentiation and function, thus leading to the development of neuroinflammatory, autoimmune diseases (Table 1).

Recently, researchers attention is pointed toward the function of ncRNAs as an another level of genetic regulation, which may contribute to MS pathogenesis. As it was shown in multiple studies, microRNAs play diverse roles in immune system, indicating that interplay between miRs and their targets is rather complex and multifactorial. What further

complicates the issue, miRs are not functioning only inside particular cell types but also they act as a signal-carrying paracrine elements contributing to cell-cell communication [138, 139].

Further studies should be conducted to reveal the role of microRNAs and other ncRNAs as they compose complex and crucial regulatory machinery, being also potential and promising targets for novel therapies.

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Research Article

Sildenafil (Viagra) Protective Effects on Neuroinflammation: The Role of iNOS/NO System in an Inflammatory Demyelination Model

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We recently demonstrated that sildenafil reduces the expression of cytokines, COX-2, and GFAP in a demyelinating model induced in wild-type (WT) mice. Herein, the understandings of the neuroprotective effect of sildenafil and the mediation of iNOS/NO system on inflammatory demyelination induced by cuprizone were investigated. The cerebella of iNOS$^{-/-}$ mice were examined after four weeks of treatment with cuprizone alone or combined with sildenafil. Cuprizone increased GFAP, Iba-1, TNF-α, COX-2, IL-1β, and IFN-γ expression, decreased expression of glutathione S-transferase pi (GSTpi), and damaged myelin in iNOS$^{-/-}$ mice. Sildenafil reduced Iba-1, IFN-γ, and IL-1β levels but had no effect on the expression of GFAP, TNF-α, and COX-2 compared to the cuprizone group. Sildenafil elevated GSTpi levels and improved the myelin structure/ultrastructure. iNOS$^{-/-}$ mice suffered from severe inflammation following treatment with cuprizone, while WT mice had milder inflammation, as found in the previous study. It is possible that inflammatory regulation through iNOS-feedback is absent in iNOS$^{-/-}$ mice, making them more susceptible to inflammation. Sildenafil has at least a partial anti-inflammatory effect through iNOS inhibition, as its effect on iNOS$^{-/-}$ mice was limited. Further studies are required to explain the underlying mechanism of the sildenafil effects.

1. Background

Nitric oxide (NO) is a highly reactive molecule with a range of physiological functions [1, 2]. This messenger plays an important role in the modulation of vascular tone [3], neurotransmission [4, 5], and immune system [6–8]. NO is produced from L-arginine by NO synthases (NOSs). In addition to the constitutive forms of the enzyme (endothelial (eNOS or NOS3) and neuronal (nNOS or NOS1)), there is also an inducible form (iNOS or NOS2). This last is most commonly associated with inflammatory conditions in which NO is produced in large amounts.

There is strong evidence to suggest the involvement of iNOS in the development of neurodegenerative disease. Induction of iNOS, NO, and NO byproducts has been found in multiple sclerosis (MS) patients and animal models and correlates with disease severity and level of inflammatory infiltrate [9–12]. Interestingly, however, iNOS-deficient mice developed a more severe MS model [13, 14]. It was found that the elimination of iNOS does not improve, and may actually aggravate, demyelination in the cuprizone-demyelinating model [15], which suggests that the iNOS/NO system may be neuroprotective. More information is required to understand

the role of iNOS/NO in inflammatory response, oligodendrocyte cell death, and myelin damage/loss.

The cyclic guanosine $3',5'$-monophosphate (cGMP) signaling pathway is an important NO-signaling molecule. NO binds to soluble guanylyl cyclase (sGC) and increases concentration of cGMP, activating signaling cascades and leading to cGMP-dependent responses [16, 17]. The cGMP signal can be terminated by the action of several phosphodiesterases (PDE) [18]. The cGMP-selective PDE5 is expressed in the cardiovascular, neural, and immune systems [18]. Studies have shown that selective PDE5 inhibitors, widely used in the treatment of erectile dysfunction in humans, such as sildenafil (Viagra; Pfizer) and vardenafil (Levitra; Bayer), raise cGMP levels in the brain and offer protective effects, improve cognition and memory [19], reduce neuronal cell death following ischemic cerebrovascular injury [20], decrease white matter damage, and regulate inflammatory responses in MS models [21, 22]. Recent studies by the authors of an MS model induced in wild-type C57BL6 mice found that sildenafil has an anti-inflammatory action, reducing levels of proinflammatory cytokines and cyclooxygenase-2 (COX-2) and protecting the myelin structure [22]. However, the mechanism of sildenafil neuroprotection remains unknown.

In the present study, inflammatory demyelination was induced in iNOS$^{-/-}$ mice, and sildenafil was administered for four weeks. The focus of this study was to identify the role of a potent inflammation-associated molecule, iNOS-derived NO, in protective mechanisms related to sildenafil. The present study also aimed to clarify the role of NO protective and/or deleterious mechanisms in the demyelinating model.

2. Materials and Methods

2.1. Experimental Design. Five iNOS knockout (B 6.129 P2-Nos2) mice, aged 7 to 10 weeks, weighing 15 to 20 g, were used per group. The mice were examined for health status, acclimated to the laboratory environment at 25°C and 12 h light/dark photoperiod, and housed in metal cages. The control group received standard laboratory diet and pure water. Over a four-week period, the experimental groups received either 0.2% cuprizone (oxalic-bis-cyclohexylidenehydrazide Sigma-Aldrich Inc., St. Louis, MO, USA) mixed into the chow and pure water or 0.2% cuprizone in the chow and 25 mg/kg of body weight of sildenafil (Viagra; Pfizer Inc., New York, NY, USA) administered through the drinking water [22–24]. Body weight was accessed every week and the drug concentration in the water was adjusted to maintain the dose. All experiments were carried out in compliance with ethical guidelines for animal experimentation (L-10/2010-CEUA; 05/10-CIBIO FIOCRUZ). After treatment, the animals were anaesthetized (i.m.) with ketamine (115 mg/kg) and xylazine (10 mg/kg) (Sespo Comércio e Indústria Ltda., São Paulo, SP, Brazil).

2.2. Immunofluorescence (IF). After anesthesia, the animals were transcardially perfused with physiological saline (20 mL), followed by 4% paraformaldehyde (Sigma-Aldrich) (40 mL) in 0.1 M phosphate (sodium phosphate monobasic and dibasic heptahydrate, Sigma-Aldrich) buffered saline (PBS), pH 7.2. Cerebella were dissected and immersed in 15% sucrose overnight, followed by 30% sucrose for a second night (36 hours total). The specimens were then embedded in OCT-Tissue Tek compound (Sakura Finetek, Torrance, CA, USA) and frozen in n-hexane (Dinâmica, São Paulo, SP, Brazil) cooled with liquid nitrogen. Cryosections (8 μm thick) were permeabilized (0.3% Triton X-100) and incubated for 1 h with blocking solution (3% BSA plus 0.2% Tween 20 in Tris buffered saline). Subsequently, the sections were incubated with antibodies for Glial Fibrillar Acidic Protein (GFAP) (DakoCytomation, cat. no. ZO 334) and Iba-1 (Wako, Osaka, Japan, cat. no. 019-19741) (both 1 : 100). Sections were incubated with primary antibodies overnight and then incubated with polyclonal Cy3-conjugated secondary antibodies (Jackson, cat. no. 705-165-147) against rabbit immunoglobulin (1 : 200) for 1 h. The slides were washed and mounted in fluorescent Prolong Gold Antifade medium (Life Technologies, cat. no. P36930) for observation under an inverted fluorescence microscope (Zeiss MicroImaging GmbH) equipped with a camera (Zeiss AxioCam MRM) and the Release 4.7.2 image analysis software.

2.3. Immunohistochemistry (IH). After perfusion as described for IF, cerebella were immediately removed and postfixed in the same fixative overnight. The samples were dehydrated in an ethanol series (Isofar Chemical Co., RJ, Brazil), cleared in xylene, and embedded in paraffin (Merck, catalog no. 1071642504). Sections (5 μm thick) were cut on an RM 2035 microtome (Reichert S, Leica), rehydrated, washed in 0.05 M PBS, and incubated in this buffer with 1% bovine serum albumin (BSA, fraction V) (Miles, Naperville, IL, USA) for one hour. Endogenous peroxidase was blocked and antigen retrieval was performed, pretreating the sections with 20 mM citrate buffer, pH 6.0, at 100°C, for 30 min. All groups were incubated with the rabbit polyclonal anti-COX-2 (Abcam, Canada/US, cat. no. ab15191) (1 : 100, overnight at 4°C). After washing, the sections were overlaid for 1 h with a biotin-conjugated secondary antibody using an HRP kit (DakoCytomation, CA, USA, Biotinylated Link Universal HRP; cat. no. K0690) and visualized with $3'$-3-diaminobenzidine (DAB) as the chromogen. The sections were then weakly counterstained with Harris' hematoxylin and mounted in entellan (Merck, cat. no. 1079610100).

2.4. Western Blotting (WB). Cerebella were quickly dissected, and each group was homogenized in an extraction cocktail (10 mM EDTA, Amresco, Solon, USA; 2 mM phenylmethane sulfonyl-fluoride, 100 mM NaF, 10 mM sodium pyrophosphate, 10 mM NaVO$_4$, 10 μg of aprotinin/mL, and 100 mM Tris, pH 7.4). Cerebella of five animals were mixed and homogenized to form a pool from each group. The WB was done in accord with Nunes et al. [22]. Briefly, the proteins (40 μg total) were separated on 6.5% (IFN-γ), 10% (TNF-α, IL-1β), or 12% (GFAP, eNOS) acrylamide gel. After overnight in 5% nonfat milk, the primary antibodies against GFAP (1 : 10,000, DakoCytomation, cat. no. ZO 334), COX-2 (1 : 1000, Abcam, cat. no. ab15191), TNF-α (1 : 1000,

Peprotech, NJ, USA, cat. no. 500-P64), IFN-γ (1 : 2500, Peprotech cat. no. 500P119), IL-1β (1 : 2500, GenWay Biotech, Inc., CA/USA, cat. no. 18-732-292194), GST3/GSTpi (1 : 250, Abcam, Canada, cat. no. ab53943), and eNOS (1 : 1000, BD Biosciences, cat. no. 610299) were incubated for four hours followed by horseradish peroxidase-conjugated antibodies anti-rabbit (1 : 80,000, Sigma-Aldrich, cat. no. A9169), anti-mouse (1 : 1,000, Sigma-Aldrich, cat. no. A0168), or anti-goat secondary antibody (1 : 100,000, Sigma-Aldrich, cat. no. A5420). For quantification, the pixel density of each band was determined using the Image J 1.38 software (http://rsbweb.nih.gov/ij/download.html; developed by Wayne Rasband, NIH, Bethesda, MD, USA). For each protein investigated, the results were confirmed in three sets of experiments. Immunoblotting for β-actin (1 : 1,000, Sigma-Aldrich, cat. no. A2228) was performed as a control.

2.5. Luxol Fast-Blue (LFB).

After perfusion as described for IF, the samples were dehydrated in a graded ethanol series, cleared in xylene, and embedded in paraffin. Sections (5 μm thick) were cut on an RM 2035 microtome (Reichert S, Leica). Myelin was detected using Luxol-Fast Blue (LFB) staining (Solvent Blue 38; Sigma-Aldrich) in accord with Nunes et al. [22]. The sections were observed under an inverted microscope (Zeiss MicroImaging GmbH) equipped with a camera (Zeiss AxioCam MRM) and Release 4.7.2 image analysis software (Zeiss).

2.6. Transmission Electron Microscopy (TEM).

After anesthesia, the animals were sacrificed by transcardial perfusion with physiological saline (20 mL), followed by 40 mL of fixative—2.5% glutaraldehyde and 4% paraformaldehyde in 0.1 M sodium cacodylate acid (Sigma-Aldrich) buffer, pH 7.2. Cerebella were quickly dissected and postfixed in the same fixative overnight. Next, cerebellum fragments were washed twice in the same buffer and postfixed in a solution containing 1% osmium tetroxide (Sigma-Aldrich), 2 mM calcium chloride, and 0.8% potassium ferricyanide (Sigma-Aldrich) in 0.1 M cacodylate buffer, pH 7.2, dehydrated in acetone and embedded in SPIN-PON resin (Embed 812-Electron Microscopy Science, Washington, PA, USA). Resin polymerization was performed at 60°C for 3 days. Semithin sections (0.5 μm in thickness) were placed on glass slides, stained with toluidine blue. Ultrathin sections (70 nm in thickness) were placed on 300-mesh nickel grids, counterstained with 5% uranyl acetate (Electron Microscopy Science) and lead citrate (Sigma-Aldrich), and examined using a FEI Morgagni 268D transmission electron microscope.

2.7. Statistical Analysis.

The densitometric values of the immunoreactive bands (immunoblotting) were analyzed using the GraphPad Prism software package (San Diego, CA, USA). One-way analysis of variance (ANOVA), followed by Dunnett's and/or Tukey's posttest, was used to compare groups. The results were expressed as means ± SE, when appropriate. A P value < 0.05 indicated statistical significance.

3. Results

Clinical analysis revealed that cuprizone-treated animals suffered from motor limitations, such as tremors, and abnormal walking and posture. The group treated with sildenafil (25 mg/kg) exhibited normal walking and posture, and tremors were either mild or nonexistent.

The clinical signs were observed and recorded by three observers. The iNOS$^{-/-}$ control animals exhibited normal motor function and posture and explored their environment normally. This group was classified as score 0 (no sign). The mice treated with cuprizone exhibited arched (shortened) posture and tremors and had difficulty in exploring the environment. This group was classified as score 2. Mice treated with 25 mg/kg of sildenafil both walked and were able to explore the environment normally, with no or mild tremors, and were classified as score 1.

Cuprizone increased GFAP, TNF-α, and COX-2 expression in cerebellum, indicating astrocyte activation (reactive gliosis) and neuroinflammation. Sildenafil treatment did not reduce the levels of these proteins in mice without iNOS.

Western Blotting (WB) analysis showed that GFAP, a marker of astrocyte activation (reactive gliosis), was present in the cerebellum of untreated iNOS$^{-/-}$ animals (control) (Figure 1(a)). Treatment with 0.2% cuprizone for four weeks significantly increased expression of this protein (Figures 1(a) and 1(c); $P < 0.001$). Animals that concomitantly received sildenafil (25 mg/Kg) and cuprizone also exhibited a high level of GFAP in comparison to control (Figures 1(a) and 1(c); $P < 0.001$).

Immunofluorescence (IF) analysis of GFAP in the cerebellum revealed the expression and location of this cytoskeletal intermediate filament protein in the animals. In the molecular layer of the cerebellum, GFAP labeling revealed long astrocytic processes with a typical arrangement that was perpendicular to the pia mater membrane (Bergmann glia) (Figure 2(e)). Astrocytic processes were also seen around other cells and vessels (arrowheads in Figures 2(b), 2(d), and 2(e)). The control group showed basal expression of GFAP localized normally (Figures 2(a) and 2(d)). Treatment with CPZ induced astrogliosis, increasing the intensity of labeling in the astrocytes (Figures 2(b) and 2(e)). In the sildenafil group, GFAP labeling was more intense than for control (Figures 2(c) and 2(f)).

The iNOS$^{-/-}$ control group had a basal level of TNF-α (Figure 3(a)). WB analysis showed that cuprizone induced a significant increase of this cytokine, indicating neuroinflammation (Figures 3(a) and 3(b); $P < 0.05$, compared to control group). Animals that received cuprizone plus sildenafil also had a significant increase of TNF-α, compared to control ($P < 0.01$).

COX-2 was analyzed by WB and immunohistochemistry (IH) tests. This enzyme was expressed in a minimal amount in the cerebellum of the iNOS$^{-/-}$ control group (Figures 3(c) and 4(a)). CPZ administration induced a significant increase in COX-2 expression, compared to control (Figures 3(c) and 3(d); $P < 0.01$). Sildenafil treatment did not decrease COX-2, which remained high in comparison with the control group

(a)

(b)

(c)
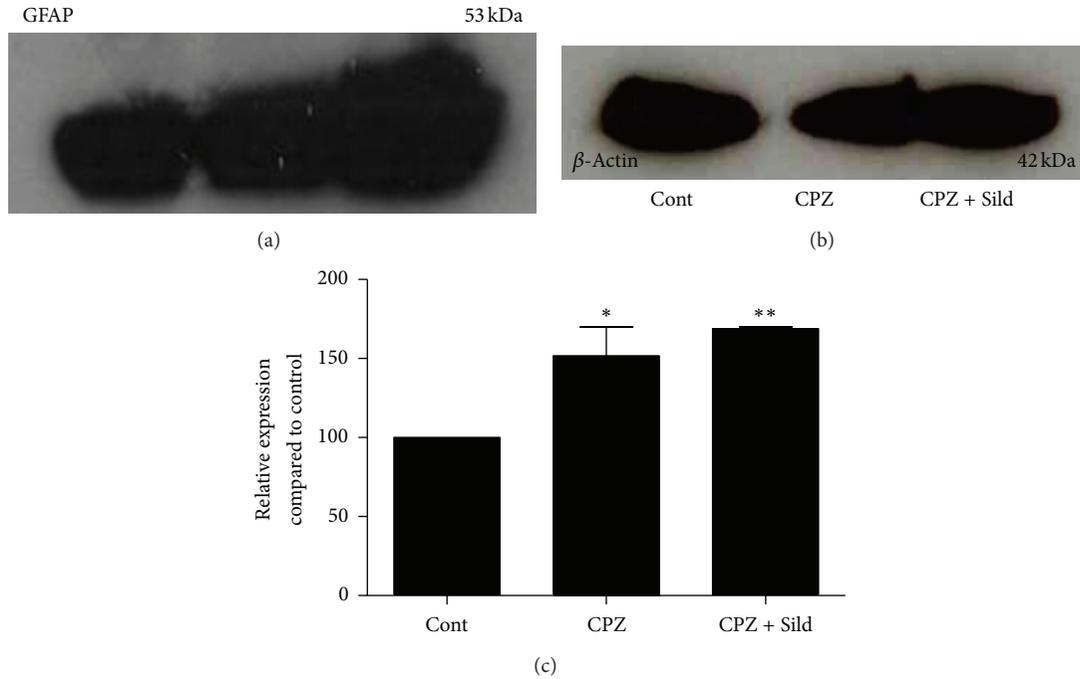
Figure 1: Western blotting for GFAP. (a) GFAP immunoblot of iNOS$^{-/-}$ control (Cont), cuprizone (CPZ), and cuprizone plus sildenafil (CPZ + Sild) groups. (b) β-actin immunoblot. (c) Graph represents quantification and statistical analysis. The control group showed basal expression of GFAP. CPZ treatment induced a significant increase of this protein and sildenafil plus CPZ did not reduce GFAP expression, which remained higher in relation to the control group. The experiment was performed in triplicate ($n = 5$ animals/group). The results were expressed as mean ± SE. $^*P < 0.05$, $^{**}P < 0.01$ compared to control.

(a)

(b)

(c)

(d)

(e)

(f)
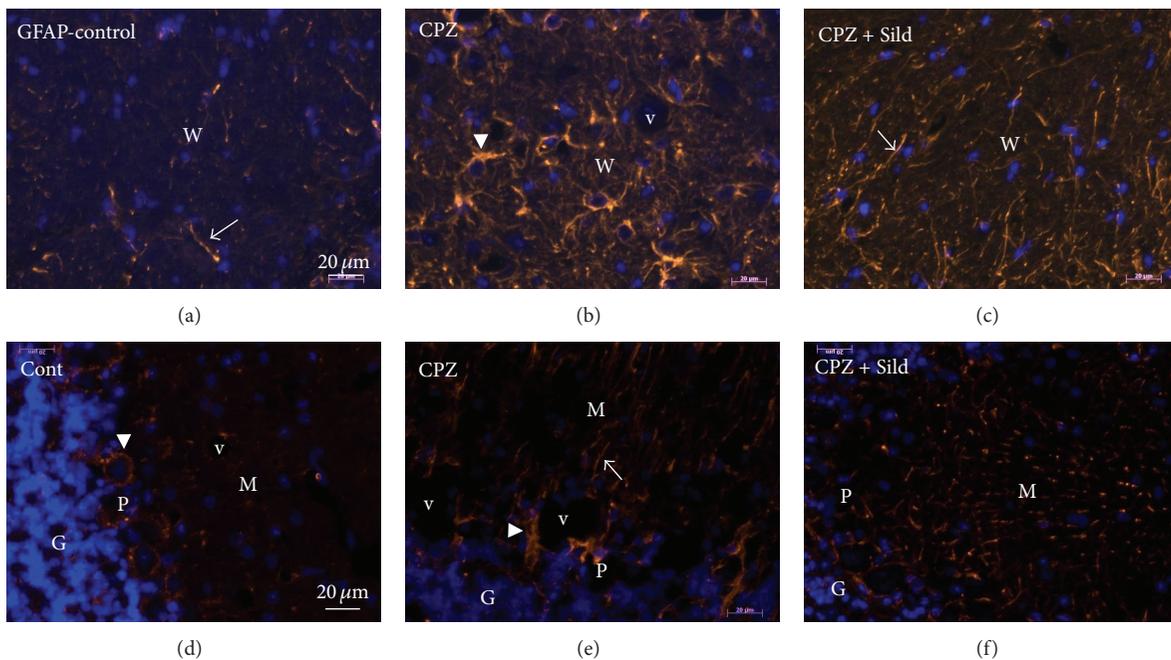
Figure 2: Immunofluorescence for GFAP. (a) and (d) show expression and physiological locations of GFAP in mice without iNOS and without treatment. CPZ administration ((b), (e)) induced reactive gliosis, with thicker and more numerous astrocytic processes, compared to control. GFAP remained high in relation to control, after application of sildenafil plus CPZ ((c), (f)). Arrows show astrocytic processes and arrowheads point to processes around vessels and other cells. W: white matter, M: molecular layer, P: purkinje layer, G: granular layer, and v: vessel. Bars: 20 μm.

(a)

(c)

(b)

(d)
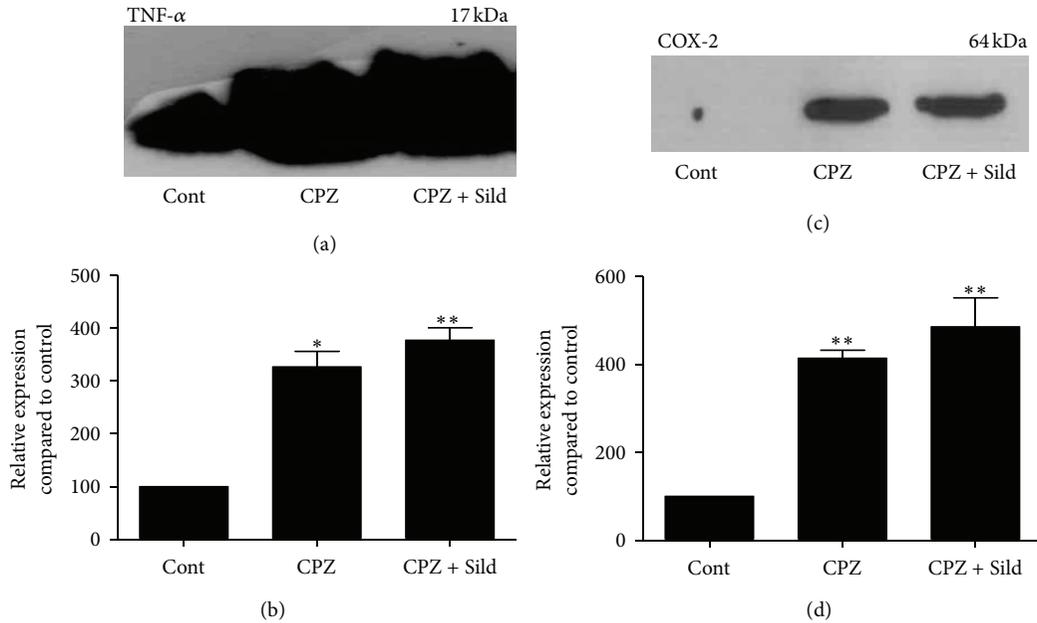
FIGURE 3: Western blotting for TNF-α and COX-2. ((a), (c)) Immunoblots of iNOS$^{-/-}$ control (Cont), cuprizone (CPZ), and cuprizone plus sildenafil (CPZ + Sild) groups. ((b), (d)) Graphs represent quantification and statistical analysis. The control group showed basal expression of TNF-α. CPZ treatment caused a significant increase of this cytokine, and sildenafil plus CPZ did not decrease its expression, which remained higher in relation to the control group. Only minimum amounts of COX-2 were present in the control group. CPZ and CPZ + Sild caused a significant increase of this enzyme, compared to control. The experiment was performed in triplicate ($n = 5$ animals/group). The results were expressed as mean ± SE. $^{*}P < 0.05$, $^{**}P < 0.01$ compared to control.


(a)

(b)

(c)

(d)

(e)

(f)
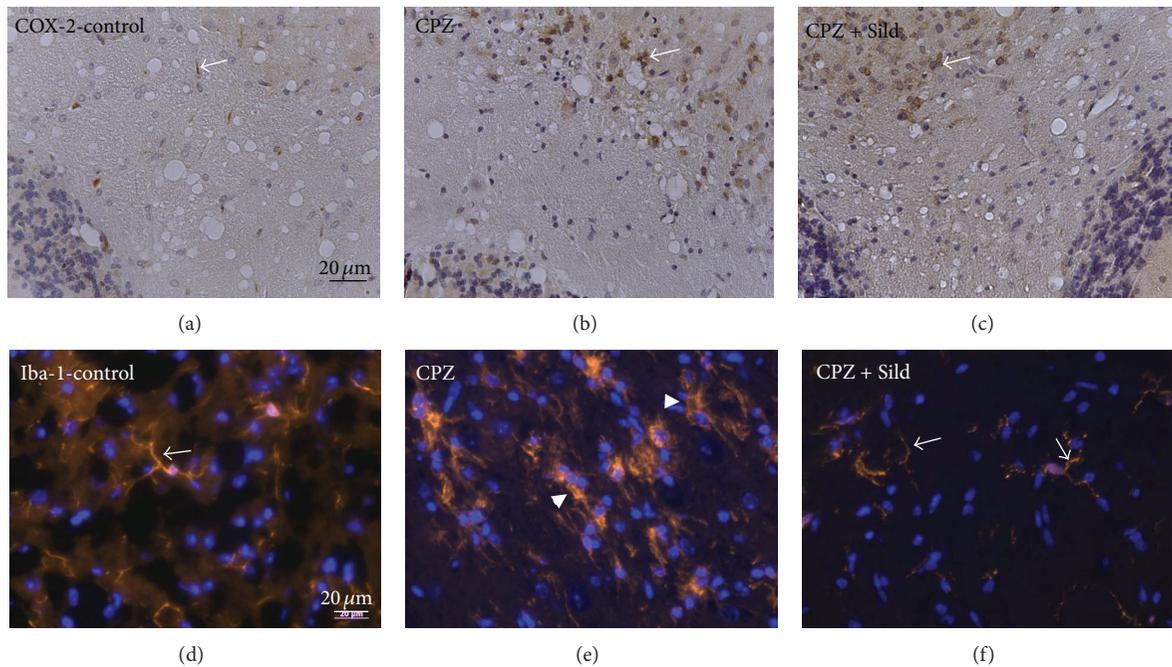
FIGURE 4: Immunohistochemistry for COX-2 ((a)–(c)) and immunofluorescence for Iba-1 ((d)–(f)). iNOS$^{-/-}$ control (a) showed very low COX-2 expression (arrow). After CPZ treatment (b), COX-2 labeling significantly increased, mainly in white matter, in relation to control. Animals treated with CPZ + Sild (c) also increased COX-2, comparing to the control group. A basal expression of Iba-1 was seen in control animals without iNOS (d). CPZ increased Iba-1 and induced an activated phenotype (arrowheads) of microglia (e). Sildenafil plus CPZ decreased Iba-1 and induced latent phenotype (arrows) of microglia (f).

IL-1β 17 kDa

Cont   CPZ   CPZ + Sild

(a)

IFN-γ 17 kDa

Cont   CPZ   CPZ + Sild

(c)

(b)

(d)
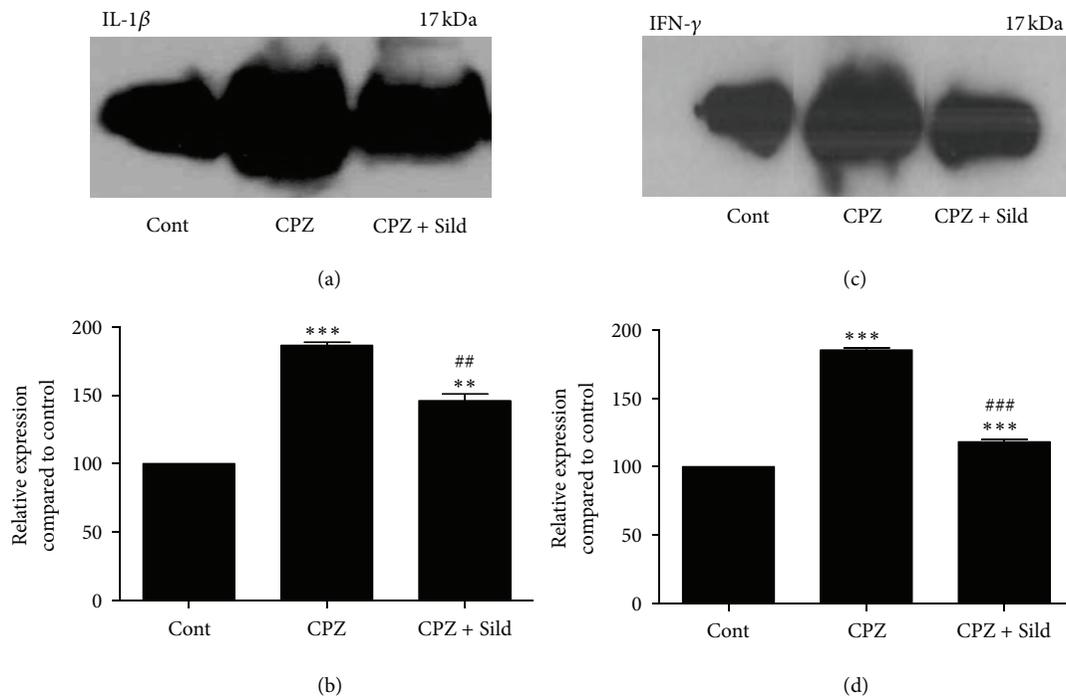
FIGURE 5: Western blotting for IL-1β and IFN-γ. ((a), (c)) Immunoblots of iNOS$^{-/-}$ control (Cont), cuprizone (CPZ), and cuprizone plus sildenafil (CPZ + Sild) groups. ((b), (d)) Graphs represent quantification and statistical analysis. The control group showed basal expression of IL-1β and IFN-γ. CPZ treatment induced a significant increase of these cytokines, compared to control. Sildenafil plus CPZ significantly decreased IL-1β and IFN-γ expression, in relation to the CPZ group. The experiment was performed in triplicate. The results were expressed as mean ± SE. $^{**}P < 0.01$, $^{***}P < 0.001$, compared to control; $^{##}P < 0.01$, $^{###}P < 0.001$, compared with CPZ.

(Figures 3(c) and 3(d); $P < 0.01$). IH labeling revealed the expression and location of COX-2 (Figures 4(a)–4(c)), which was mainly found in the white matter of the cerebellum. The control group had a very low expression of this enzyme (Figure 4(a)), while treated animals had a high expression of COX-2 in white matter (Figures 4(b) and 4(c)).

Immunoblot control with β-actin is shown in Figure 1(b).

Cuprizone increased Iba-1, IL-1β, and IFN-γ expression in the cerebellum of iNOS$^{-/-}$ mice. While sildenafil decreased the expression of these proteins, the level of expression remained above that of control animals.

The microglial marker, Iba-1, was analyzed by IF. There was physiological expression of Iba-1 in the cerebellum of iNOS$^{-/-}$ control animals, with cells with processes that were typically branched, thin and weakly labeled (arrow, Figure 4(d)). Cuprizone treatment induced a stronger expression of Iba-1 (Figure 4(e)), compared to the control group, with thicker and more intensely labeled microglial processes. These processes lost their typical thin branched appearance, indicating that the cell phenotype had acquired activated characteristics. Sildenafil together with CPZ decreased Iba-1 expression, and the microglia exhibited thin, highly branched processes, typical of a latent state (Figure 4(f)).

Immunoblotting for IL-1β and IFN-γ revealed basal expression of these cytokines in the iNOS$^{-/-}$ control group (Figures 5(a) and 5(c)). Cuprizone strongly increased the expression of these cytokines (both $P < 0.001$), compared to the control group (Figures 5(a)–5(d)), which indicates

neuroinflammation. Sildenafil treatment resulted in a significant decrease of IL-1β and IFN-γ compared to the cuprizone group ($P < 0.01$). However, the levels of IL-1β and IFN-γ remained higher in the sildenafil-treated group when compared with baseline levels of the control group ($P < 0.01$ and $P < 0.001$, resp.).

Mice without iNOS underwent a significant increase in eNOS expression, compared to wild-type animals.

Untreated mice iNOS$^{-/-}$ (control group; $P < 0.01$) and iNOS$^{-/-}$ animals treated with CPZ or CPZ + Sild (both $P < 0.001$) showed a significant increase in eNOS levels, compared with wild-type mice that did not undergo treatment. The expression of eNOS in iNOS$^{-/-}$ mice treated with CPZ and CPZ plus sildenafil was not significantly higher when compared to iNOS$^{-/-}$ control animals but showed a propensity to increase (Figures 6(a) and 6(b)).

Cuprizone decreased GSTpi, indicating the depletion of mature oligodendrocytes. Sildenafil increased GSTpi expression in the cerebellum of iNOS$^{-/-}$ mice.

GSTpi, a marker of myelinating oligodendrocytes, was expressed in the cerebellum of the control group (Figure 7(a)). After treatment with CPZ, this marker decreased significantly ($P < 0.001$) compared to the control group, suggesting an impairment of mature oligodendrocytes and consequent myelin damage. Sildenafil treatment together with CPZ induced partial recovery of the GSTpi marker, indicating that sildenafil had a protective effect on mature
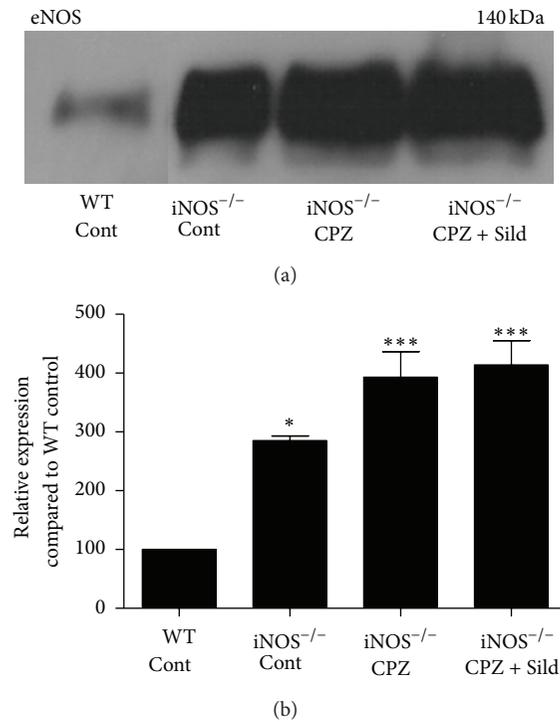
FIGURE 6: Western blotting for eNOS. (a) Immunoblot of wild-type mice control (WT cont), iNOS$^{-/-}$ mice control (iNOS$^{-/-}$ cont), iNOS$^{-/-}$ animals treated with cuprizone (iNOS$^{-/-}$ CPZ), and iNOS$^{-/-}$ animals treated with cuprizone plus sildenafil (iNOS$^{-/-}$ CPZ + Sild). (b) Graph represents quantification and statistical analysis. eNOS was physiologically expressed in WT mice. Animals without iNOS without treatment showed a significant increase of this enzyme, compared to WT control. After CPZ and CPZ plus sildenafil, iNOS$^{-/-}$ animals also showed a significant increase of eNOS, compared to WT animals, but no significant difference was identified between iNOS$^{-/-}$ control and treated animals. The experiment was performed in triplicate. The results were expressed as mean ± SE. $^*P < 0.05$, $^{***}P < 0.001$, compared to control.

FIGURE 7: Western blotting for GSTpi. (a) Immunoblot of iNOS$^{-/-}$ control (Cont), cuprizone (CPZ), and cuprizone plus sildenafil (CPZ + Sild) groups. (b) Graph represents quantification and statistical analysis. iNOS$^{-/-}$ control showed basal expression of GSTpi. After CPZ, GSTpi decreased significantly, compared to iNOS$^{-/-}$ cont. CPZ + Sild treatment increased GSTpi expression in relation to the CPZ group, but the levels of this protein remained significantly decreased compared to control. The experiment was performed in triplicate. $^{***}P < 0.001$ compared to control; $^{###}P < 0.001$ compared to CPZ.

oligodendrocytes. In the sildenafil group, GSTpi decreased in comparison with control ($P < 0.001$) but increased in comparison to the cuprizone group ($P < 0.001$; Figures 7(a) and 7(b)).

Myelin sheath structure was disorganized in animals without iNOS which did not undergo treatment (control). Cuprizone induced more severe myelin disruption. Sildenafil improved myelin structure and ultrastructure.

Interestingly, iNOS$^{-/-}$ animals without treatment (control) had moderate disorganization of the myelin structure and ultrastructure (Figures 8(a), 8(d), and 8(g)). Standard LFB staining showed that the control group iNOS$^{-/-}$ had vacuoles in white matter (arrow in Figure 8(a)), resulting in inhomogeneous and disorganized tissue. Qualitative analysis of ultrathin cerebellum sections by TEM revealed that the myelin sheath was often shredded, with blanks, and without the characteristic lamellar pattern (arrows in Figures 8(d) and 8(g)).

Cuprizone-treated animals exhibited more severe damage to myelin structure and ultrastructure (Figures 8(b), 8(e), and 8(h)). LFB staining (Figure 8(b)) showed vacuoles (arrows) and spaces (arrowhead) as slits in white matter, characteristic of highly disorganized tissue. TEM revealed that CPZ caused serious damage to the myelin sheath ultrastructure, which had numerous spaces between the shreds in practically all fibers (represented by arrows in Figures 8(e) and 8(h)). The typical lamellar pattern was entirely absent.

Simultaneous treatment with sildenafil and CPZ resulted in a noticeable improvement of myelin organization (Figures 8(c), 8(f), and 8(i)). White matter was more homogeneous, presenting fewer and, in general, smaller vacuoles and slit-like spaces (Figure 8(c)). Ultrathin section analysis revealed a more preserved myelin sheath which rarely showed signs of shredding.

4. Discussion

The cuprizone model is characterized by primary and reversible demyelination, due to peripheral immune system-independent myelin injury [25]. In this model, demyelination is accompanied by a well-characterized sequence of events involving the depletion of mature oligodendrocytes, microglia activation, and astrocyte proliferation [26]. Therefore, demyelination and resident neuroinflammation induced
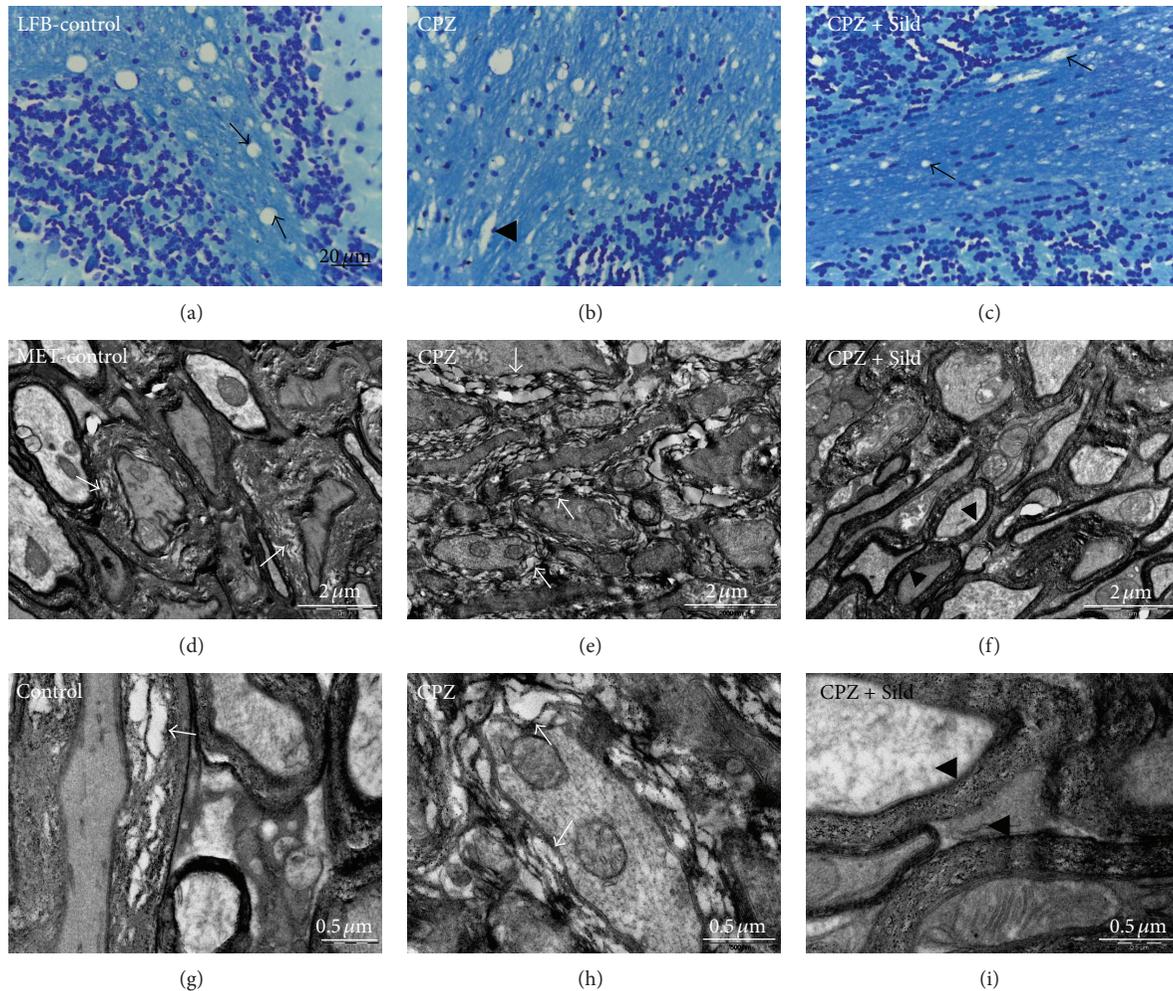
FIGURE 8: Luxol Fast Blue (LFB) staining ((a)–(c)) and electron micrographs ((d)–(i)). (a), (d), and (g) represent iNOS$^{-/-}$ control group; (b), (e), and (h) represent CPZ-treated animals; (c), (f), and (i) represent CPZ + Sild-treated animals. Arrows in (a), (c): vacuoles in the white matter; arrowhead in (b): spaces between fibers; arrows in (d), (e), (g), and (h): damaged myelin sheath; arrowheads in (f), (i): preserved myelin. Bars = 20 μm ((a)–(c)); 2 μm ((d)–(f)); 0.5 μm ((g)–(i)).

by cuprizone in rodents have been widely used as a model for MS [20, 27]. In the present study, the usefulness of the cuprizone model is considerable as it allows evaluation of the role of the iNOS/NO-sGC-cGMP pathway in the inflammation and demyelination mediated by resident CNS cells.

The cerebellum was chosen for analysis, as it is an important CNS affected region in MS patients, revealing severe white matter atrophy [28, 29]. Furthermore, the authors of the present study had recently investigated this region [22]. The present data, relating to iNOS$^{-/-}$ animals, will be discussed with reference to this previous study. Nunes et al. (2012) [22] showed that in C57BL/6 wild-type (WT) mice cuprizone induced tissue damage, increased GFAP, Iba-1, and COX-2 expression, and caused demyelination, in comparison to the control group. However, cuprizone did not affect the expression of cytokines (TNF-α, IFN-γ, IL-1β, and IL-2) in WT mice. In the previous study by the authors, sildenafil

reduced GFAP and Iba-1 expression in comparison to the cuprizone group, preserved myelin and axon ultrastructure, and significantly downregulated IFN-γ, TNF-α, IL-1β, IL-2, and COX-2 expression in comparison to the control and/or cuprizone groups. The previous results demonstrated the protective effect of sildenafil on the cerebellum. Sildenafil promotes the accumulation of cGMP, which is the main NO signaling molecule. It was considered important to investigate the role of NO in the MS-model and the effects of sildenafil more profoundly. Therefore, in the present study, the relationship between iNOSnull mice, the MS-model, and the effects of sildenafil in the cerebellum was investigated.

In the absence of iNOS/NO, cuprizone significantly increased expression of GFAP, TNF-α, COX-2, Iba-1, IL-1β, and IFN-γ. In addition, cuprizone intoxication decreased GSTpi, a marker for myelinating oligodendrocytes, damaged myelin, and induced tremors, abnormal walking, and posture. This data indicates that cuprizone intoxication occurred

even without the iNOS-NO system and was stronger than in WT mice, where cuprizone did not increase cytokine expression [22]. Interestingly, iNOS$^{-/-}$ control animals showed an altered myelin structure.

It was hypothesized that, in the absence of iNOS, eNOS may be overexpressed as a compensatory mechanism. Bernardini et al. [30] showed that treatment with endotoxin influenced NOS expression, upregulating iNOS and, simultaneously, downregulating eNOS. There appeared to be a regulatory relationship between the expression of iNOS and eNOS. In fact, it was found here that eNOS was strongly expressed in iNOS$^{-/-}$ animals in comparison with WT mice. The eNOS levels remained high after the administration of cuprizone and cuprizone plus sildenafil. In normal conditions, low levels of NO produced by both eNOS and nNOS participate in cell signaling and regulate physiologic processes [31]. However, eNOS overexpression (and consequently, constant high concentration of NO) can be responsible for myelin changes and proinflammatory susceptibility in iNOS$^{-/-}$ animals.

On the other hand, iNOS possesses an important feedback mechanism in inflammatory conditions, when the increase of this enzyme is self-regulated, and induces a reduction of some proinflammatory proteins [32–35]. The inhibition of iNOS activity induces enhancement of IL-1β, IL-6, and TNF-α levels [36] and, subsequently, a persistent increase of iNOS expression, downregulating the TNF receptor [35]. In the present study, absence of iNOS may explain increased TNF-α, IFN-γ, and IL-1 after cuprizone treatment in iNOS$^{-/-}$ mice, while cuprizone did not increase these cytokines in WT mice [22]. This feedback mechanism is coregulated by a high concentration of cGMP [32]. Therefore, another hypothesis for explaining the more severe inflammation induced by cuprizone in mice without iNOS is the absence of iNOS feedback mechanism.

Interestingly, although sildenafil had a low anti-inflammatory effect on iNOS$^{-/-}$ mice, it considerably improved the myelin structure of mice without iNOS. Cuprizone is a copper chelator which leads to direct oligodendrocyte death with subsequent demyelination [37]. In this model, oligodendrocyte death and demyelination are independent of immune and inflammatory response. It was found that cGMP analog (8-Br-cGMP) protects differentiated oligodendrocytes from death initiated by staurosporine, thapsigargin, or kainate [38]. It is possible that sildenafil, through the accumulation of cGMP, has a direct beneficial effect on oligodendrocytes, protecting these cells and improving myelination, independent of its anti-inflammatory effects.

In conclusion, the findings of the present study show that iNOS$^{-/-}$ mice are more susceptible to cuprizone intoxication due to the potential involvement of two mechanisms: (1) iNOS-negative feedback mechanism in inflammatory conditions is absent and, consequently, proinflammatory proteins, such as cytokines and COX-2, are excessively increased; (2) eNOS is overexpressed by a compensatory mechanism and generates chronically high levels of NO, damaging the tissue. Also, the results of the present study suggest that sildenafil may exert its anti-inflammatory effects mainly through iNOS inhibition, by cGMP-iNOS feedback. In addition, sildenafil may have a direct protective effect on oligodendrocytes. Further studies are required to explain the molecular mechanism of sildenafil protection in the central nervous system.

Conflict of Interests

The authors declare that they have no conflict of interests.

Authors' Contributions

Catarina Raposo and Ana Karolina Santana Nunes were involved in the treatment of animals, development of WB, IF and MET testing procedures, analysis and interpretation of results, and the drafting of the paper. Catarina Raposo and Ana Karolina Santana Nunes contributed equally to the development of this study. Rayana Leal de Almeida Luna and Shyrlene Meiry da Rocha Araújo contributed to the treatment of animals, development of IH and LFB testing procedures, and analysis and interpretation of results. Maria Alice da Cruz-Höfling contributed to the analysis and interpretation of data and paper revision. Christina Alves Peixoto contributed to the supervision of technical procedures, the analysis and interpretation of data, and paper revision and gave final approval of the version to be published.

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Review Article

Development and Treatments of Inflammatory Cells and Cytokines in Spinal Cord Ischemia-Reperfusion Injury

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During aortic surgery, interruption of spinal cord blood flow might cause spinal cord ischemia-reperfusion injury (IRI). The incidence of spinal cord IRI after aortic surgery is up to 28%, and patients with spinal cord IRI might suffer from postoperative paraplegia or paraparesis. Spinal cord IRI includes two phases. The immediate spinal cord injury is related to acute ischemia. And the delayed spinal cord injury involves both ischemic cellular death and reperfusion injury. Inflammation is a subsequent event of spinal cord ischemia and possibly a major contributor to spinal cord IRI. However, the development of inflammatory mediators is incompletely demonstrated. And treatments available for inflammation in spinal cord IRI are insufficient. Improved understanding about spinal cord IRI and the development of inflammatory cells and cytokines in this process will provide novel therapeutic strategies for spinal cord IRI. Inflammatory cytokines (e.g., TNF-α and IL-1) may play an important role in spinal cord IRI. For treatment of several intractable autoimmune diseases (e.g., rheumatoid arthritis), where inflammatory cytokines are involved in disease progression, anti-inflammatory cytokine antagonist is now available. Hence, there is great potential of anti-inflammatory cytokine antagonist for therapeutic use of spinal cord IRI. We here review the mediators and several possibilities of treatment in spinal cord IRI.

1. Inflammation in Spinal Cord IRI

Inflammation is a subsequent event of spinal cord ischemia and a plausible pathway in spinal cord ischemia-reperfusion injury (IRI) [1]. A series of metabolic processes ensue following ischemia. In a porcine model of 45-minute thoracoabdominal aortic occlusion, a strong immune response, which manifested as hyperemia and accumulation of inflammatory cells, occurred 48 h after the end of the aortic occlusion [2]. In a rabbit model of spinal cord ischemia, prominent inflammatory cell infiltration was observed [3]. These studies indicate that ischemia initiates an inflammatory reaction in the spinal cord.

Restoration of spinal cord blood flow would lead to so called reperfusion injury [4], which has been known as a biochemically mediated event [5]. Restored blood flow stimulates expression of adhesion molecules and chemokines, resulting in inflammatory reaction that involves neurotoxicity, recruitment of leucocytes, polymorphonuclear microvessel endothelial damage, hypoperfusion, and apoptosis [6]. In a swine model of spinal cord IRI, neutrophil sequestration and neuronal viability changed within 24 hours of reperfusion [7]. In a rat model of spinal cord IRI, researchers observed inflammatory cell infiltration in the gray matters of the spinal cords [1]. Delayed motor neuron death was detected during the same period as the strong immune response in the gray matter [2]. However, in a murine model of thoracic aortic ischemia reperfusion, there was no correlation between markers of inflammation and neurologic outcomes [8]. These observations indicated that inflammation might be a major contributor to spinal cord IRI, especially in the reperfusion period.

2. Inflammatory Cells in Spinal Cord IRI

Inflammatory response in spinal cord IRI was characterized by a massive accumulation of inflammatory cells in the gray matter [2]. Inflammatory cells in spinal cord IRI mainly include macrophages, lymphocytes, neutrophils, microglia, and astrocytes [9]. They were usually observed as perivascular infiltration cells in spinal cord IRI [9]. Kiyoshima T demonstrated that delayed onset paraplegia was largely associated with necrotic cell death with prominent inflammatory cell infiltration [10]. However, little is known about the activation and reaction of these inflammatory cells in spinal cord IRI.

2.1. Macrophages. In a rat model of spinal cord IRI, a number of bone marrow-derived macrophages were present 7d after IRI [11]. In the animals that suffered from severe paraplegia, a robust accumulation of bone marrow-derived macrophages occupied the entire ischemic gray matter [11]. In a rabbit model of spinal cord IRI, macrophages were first detected at 8 hours after reperfusion and mainly surrounded the infarction area [12].

2.2. Neutrophils. Activated neutrophils play a key role in the development of spinal cord IRI [13]. Accumulation of neutrophils in the postischemic spinal cord tissue could be evaluated by measuring myeloperoxidase (MPO) levels. In a rat model of spinal cord IRI, spinal cord tissue levels of MPO were increased after spinal cord IR, peaking at 24 h after reperfusion [14]. In a rat model of spinal cord IRI, tissue MPO activity (mean 0.60 ± 0.046 U/g) increased significantly at 24 h after reperfusion, compared with the control group (mean 0.23 ± 0.040 U/g) [9].

2.3. Microglia. Microglia are the resident immune cells of the central nervous system [15]. They could be activated early after spinal cord reperfusion injury and share many immunological characteristics with peripheral macrophage [12, 15]. Researches indicate that the proliferation and activation of microglia contributes to excitotoxicity [16], which is an important mechanism of spinal cord IRI. Olson examined the immune response by microglia in the spinal cord; their observations revealed that microglia in the spinal cord of mice expressed higher levels of surface immune molecules and may have different immune reactivity which may contribute to spinal cord diseases [15].

2.4. Astrocytes. Astrocytes are one of the major components of the blood-brain (spinal cord) barrier and play a role in the development of spinal cord IRI and its neurological outcomes. In a rabbit model of spinal cord IRI, astrocytes were activated early (2 hours) after reperfusion in the gray matter of the lumbar spinal cord, but confined to the area where neurons started to show degeneration [12]. This finding suggested that astrocytes might be important in the mechanism of delayed onset motor dysfunction in spinal cord IRI.

3. Cytokines in Spinal Cord IRI

Spinal cord IRI is correlated to increases in inflammatory chemokines release [17]. Several cell types have been shown to synthesize inflammatory cytokines [1]. Experiments in animal models of spinal cord IRI revealed that macrophages and microglia strongly expressed tumor necrosis factor-α (TNF-α), interleukin 1β (IL-1β), and other mediators [7, 11, 16]. In a rat model of spinal cord IRI, strong staining of IL-1β, IL-10, and TNF-α was observed, suggesting a dramatic infiltration of inflammatory cells in both gray matters and peripheral white matters [18].

Cytokines are a group of proteins produced during the activation of inflammatory response [1] and play an important role in the subsequent spinal cord IRI. However, data about the pathways and effects of these cytokines in the spinal cord IRI are still limited [9]. In rats that suffered from severe paraplegia induced by spinal cord IRI, TNF-α, IL-1β, and other mediators were strongly expressed [11]. Lu and colleagues discovered that the mitogen-activated protein kinase/extracellular signal-regulated kinase (MEK/ERK) pathway might play a noxious role in spinal cord IRI via participating in inflammatory reactions and cytokine production [19].

Species and alternation of cytokines in spinal cord IRI have not been well determined. Smith and colleagues analyzed 23 cytokines in a mice model of spinal cord IRI, and the chemokines IL-1, IL-6, keratinocyte chemoattractant (KC; murine equivalent of human IL-8), and TNF-α increased significantly and showed a biphasic response [17]. However, Kunihara and colleagues discovered that levels of TNF-α, IL-1β, IL-6, and IL-12 in serum and cerebrospinal fluid (CSF) did not significantly change [20].

3.1. TNF-α. TNF-α is a potent activator of neutrophils [21]. TNF-α binds to two receptors: type 1 TNF receptor (p55) and type 2 TNF receptor (p75), which are expressed in many types of cells [22].

TNF-α increased significantly in reperfusion period and remained at high level after reperfusion. In a rabbit model of 30 minutes aortic occlusion and 2 hours of reperfusion, TNF-α level was significantly increased to 120.44 ± 8.95 pg/mg protein in the IRI group compared with the sham group (25.34 ± 1.03 pg/mg protein) [4].

Besides significant elevated expression, TNF-α showed trends toward a biphasic, early and late, peak in expression [17]. In a rat model of spinal cord IRI (a balloon catheter placed into the aorta), TNF-α level was significantly increased within 1.5 hours after the transient ischemia and peaked at 3 hours [21]. In a swine model of spinal cord IRI, TNF-α levels increased significantly by 6 hours to 12 hours after reperfusion, suggesting a similar pattern of bimodal release of TNF-α after IRI [7].

The high levels of TNF-α persisted for a long time after second expression peak. In a rat model of spinal cord IRI, TNF-α levels in the 24 hours sham operated group and 24 hours IRI group were 48.30 ± 11.11 pg/mL and 138.62 ± 78.58 pg/mL, respectively [1]. TNF-α levels in the 48 hours sham operated group and 48 hours IRI were 68.65 ± 25.15 pg/mL and 129.16 ± 51.27 pg/mL, respectively. There were no significant changes for TNF-α levels between the 24 hours and 48 hours after IRI ($P > 0.05$) [1]. In a rat model

of spinal cord IRI, spinal cord sections from the 48 hours reperfusion group exhibited a strong positive staining for TNF-α, mainly localized in various cells in the gray matter [23]. In a rat model of spinal cord IRI, the serum levels of TNF-α increased from 257 to 629 pg/mL at 24 hours after IRI [24]. In a rabbit model of spinal cor IRI, TNF-α expression at 1.5 hours (53.4 ± 12.3) and 3 days (92.4 ± 5.7) of reperfusion was higher than at 5 days (40.4 ± 20.1), and at 3 days it was higher than at 1.5 h [25].

Excitotoxic cell death due to glutamate release is important in the secondary injury following spinal cord ischemia [26]. Neurophysiological studies show that TNF-α can potentiate the effects of glutamatergic afferent input to produce hyperactivation of neurons [26]. These results suggest that proinflammatory cytokines, especially TNF-α might contribute to excitatory cell death in spinal cord IRI.

3.2. Interleukin 1 (IL-1).
IL-1 family cytokines include the secreted proinflammatory agonist IL-1β, IL-18, and anti-inflammatory receptor antagonist IL-1a [9]. IL-1β has been implicated in extensive inflammation and progressive neurodegeneration after ischemia [10]. In a rat model of spinal cord IRI, spinal cord sections from the 48 hours reperfusion group exhibited a strong positive staining for IL-1, mainly localized in various cells in the gray matter [23].

IL-1 also showed a biphasic expression in spinal cord IRI. In a mice model of spinal cord IRI, IL-1 expression was significantly increased at 6 hours and 36 hours into reperfusion [17]. IL-1 expression at 6 hours and 36 hours was also increased compared with 18 hours, signifying a biphasic response to reperfusion [17].

IL-1β levels increased significantly in reperfusion period and remained at high levels after reperfusion. In a rat model of spinal cord IRI, IL-1β levels in the 24 hours sham operated group and 24 hours IRI group were 22.21 ± 8.64 pg/mL and 58.01 ± 26.46 pg/mL, respectively [1]. IL-1β levels in the 48 hours sham operated group and 48 hours IRI group were 31.45 ± 16.43 pg/mL and 71.65 ± 15.90 pg/mL, respectively. There were no significant changes for IL-1β levels between 24 hours IRI group and 48 hours IRI group ($P > 0.05$) [1]. In a rat model of spinal cord IRI, the mean spinal cord IL-1 amounts were 20.38 ± 2.49 at 1 day and 19.69 ± 3.21 at 3 days [19].

MEK/ERK pathway might play a noxious role in spinal cord IRI via participating in inflammatory reactions and cytokine production [19]. In a rat model of spinal cord IRI, MEK/ERK pathway inhibition with U0126, highly selective inhibitor of both MEK1 and MEK2 (MEK1/2) [27], dramatically reduced microglia accumulation and IL-1 expression, resulting in improved neuronal survival [19]. This study suggested a role of the MEK/ERK pathway in the inflammatory responses after spinal cord IRI might be partly mediated by its inhibitory effects on microglia activation and IL-1 production [19].

3.3. Interleukin 6 (IL-6).
IL-6 is a proinflammatory cytokine induced by spinal cord IRI and increased significantly in the process of IRI. In a mice model of spinal cord IRI, IL-6 was significantly increased compared with all other time points and peaked at 36 hours, without significant increase in expression at other time points [17]. In a rabbit model of aortic occlusion and reperfusion, IL-6 levels were significantly increased to 87.40 + 5.86 pg/mg protein after IRI, compared with 11.46 + 1.09 pg/mg protein in the sham group [4].

The increase in IL-6 expression continued for hours after reperfusion. In a rat model of spinal cord IRI, IL-6 levels in the 24 hours sham operated group and 24 hours IRI group were 48.21 ± 19.79 pg/mL and 372.50 ± 134.62 pg/mL, respectively. IL-6 levels in the 48 hours sham operated group and 48 hours IRI group were 216.51 ± 74.48 pg/mL and 847.20 ± 350.28 pg/mL, respectively. These data revealed that IL-6 levels at 48 hours after IRI were significantly higher than those at 24 hours ($P < 0.05$) [1].

3.4. Interleukin 8 (IL-8).
IL-8 levels might be correlated with neurological outcomes after spinal cord IRI. Kunihara and colleagues measured levels of cytokines in perioperative serum and CSF in fifteen adult patients undergoing aortic repair. IL-8 levels in CSF peaked after operation and maintained the higher levels for 72 hours. The patients with paraplegia had the highest IL-8 levels in CSF throughout the study period [20].

The increase in IL-8 levels showed a biphasic pattern in spinal cord IRI. In a mice model of spinal cord IRI, KC (murine equivalent of human IL-8) expression peaked at 6 hours and 36 hours, though the first peak was not marked enough to meet statistical significance [17]. KC expression peaked at 36 hours, meeting statistical significance when compared with that at 18 hours of reperfusion [17]. In a rat model of spinal cord IRI (a balloon catheter placed into the aorta), IL-8 level was increased and peaked at 12 hours after the transient ischemia. The biphasic expression of inflammatory cytokines would support a bimodal mechanism of spinal cord IRI.

3.5. Interleukin 10 (IL-10).
IL-10 is a potent anti-inflammatory cytokine induced by spinal cord IRI. In a rat model of spinal cord IRI, spinal cord sections from the 48 hours reperfusion group exhibited a strong positive staining for IL-10, mainly localized in various cells in the gray matter [23]. In a model of excitotoxic spinal cord injury induced by quisqualic acid, excitotoxic injury plus IL-10 treatment resulted in a significant downregulation of IL-1β and iNOS mRNA, suggesting that IL-10 could reduce spinal cord inflammation [28]. In a rat model of spinal cord IRI, MPO activity was slightly increased in IL-10-treated group (0.34 ± 0.029 U/g) with respect to control animals, suggesting that the administration of IL-10 could decrease IRI-induced MPO activity early after spinal cord IRI [9].

3.6. mRNAs.
Inflammatory mRNAs are involved in the mediation of spinal cord IRI. In a rat model of spinal cord IRI [29], some anti-inflammatory mRNAs such as annexin A7 mRNA were potential targets of miR-323. Conversely, some inflammatory mediator mRNAs such as integrin, TNF-α, IL-1β, TNF receptor-associated factor 6, interleukin-1 receptor-associated kinase 1, and CD80 mRNAs were potential targets of miR-210, miR-146a, and miR-199a-3p, which were downregulated after spinal cord IRI [29].

4. Treatments for Inflammation in Spinal Cord IRI

There are several strategies applied for treatments of inflammation in spinal cord IRI, targeting at inflammatory cells, cytokines, and their receptors and pathways. However, most of these therapeutic strategies are insufficiently elucidated and needed further measurements.

4.1. Adenosine A_{2A} Receptor Activation. Adenosine A_{2A} receptor activation might attenuate spinal cord inflammation [7]. In a swine model of spinal cord IRI, adenosine A_{2A} receptor activation attenuates every aspect of IRI [7]. In a mice model of aortic aneurysm formation following elastase perfusion, data suggest that $A_{2A}R$ is functionally active in mediating immune cell recruitment and protease expression in the medial and adventitial layers of the aortic wall during aortic aneurysm formation [30].

Systemic ATL-146e, a selective adenosine A_{2A} agonist, has been shown to reduce paralysis after spinal cord ischemia [31]. In a rabbit model of spinal cord IRI (45-minute cross-clamping of the infrarenal aorta), ATL-146e reduced spinal cord reperfusion injury probably by reducing circulating TNF-α during a critical 3 h reperfusion interval [31].

4.2. Pentoxifylline. Pentoxifylline is an inhibitor of TNF-α. Since TNF-α is an important contributor to spinal cord IRI which might induce necrosis and apoptosis of cells, its inhibitor might exhibit a protective role in spinal cord injury following ischemia [32]. In a rabbit model of spinal cord IRI (45 minutes cross-clamping of the infrarenal aorta), a significant decrease in both necrotic and apoptotic neurons was observed in the Pentoxifylline-treated groups compared with the IRI group ($P < 0.05$) [32].

4.3. U0126. U0126 is a specific inhibitor of MAPK/ERK kinases 1/2 (MEK1/2) [27]. In a rat model of spinal cord IRI, the IL-1 levels in the U0126 group were significantly lower than those in the control group ($P = 0.021$), suggesting that MEK/ERK inhibition with U0126 might reduce microglia accumulation and IL-1 expression [19]. A further study indicated that pretreatment with U0126 inhibited ERK1/2 phosphorylation and significantly attenuated apoptosis and increased neuronal survival [27].

4.4. Infliximab. Infliximab is a humanized mouse monoclonal antibody to TNF-α [22]. In a rabbit model of spinal cord IRI, the infliximab group had significantly less vascular proliferation, edema, and neuron loss than the I/R group.

4.5. IL-1ra. IL-1ra is receptor antagonist and has anti-inflammatory properties and was expected to suppress inflammatory response in spinal cord IRI [10]. In a rabbit model of spinal cord IRI (aortic cross-clamping), a higher number of viable neurons were observed with less severe spinal cord injury in IL-1ra group ($P < 0.01$ at 24 hrs, $P < 0.05$ at 72 hrs, and $P < 0.05$ at 120 hrs). TUNEL-positive neurons were also significantly reduced by the administration of IL-1ra ($P < 0.01$ at 24 hrs and $P < 0.05$ at 120 hrs) [10]. These studies indicated that IL-1-targeted anticytokine therapy could be

a potential strategy for improving the neurological outcomes after spinal cord IRI [10].

4.6. Activated Protein C (APC). It is reported that APC might reduce spinal cord injury in rats by inhibiting neutrophil activation after the transient ischemia [21]. In a rat model of spinal cord IRI (a balloon catheter placed into the aorta), the increases in the tissue levels of TNF-α, rat IL-8, and myeloperoxidase in the ischemic part of the spinal cord were significantly reduced in animals that received APC [21].

4.7. Statins. Statins are the 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors and might have pleiotropic effects that are independent of cholesterol lowering, such as anti-inflammatory effects, antioxidant effects, and endothelial function improvement [29].

4.8. Tetramethylpyrazine (TMP). TMP is a pure compound derived from Ligusticum chuanxiong, which is widely applied for the treatment of ischemic stroke. Recent studies indicated that TMP might also exert neuroprotective effects on inflammation in spinal cord IRI. In a rat model of spinal cord IRI [23], treatment with TMP considerably reduced the degree of positive IL-1 and TNF-α staining and increased the degree of positive IL-10 staining in the spinal cord. Compared with the findings from the sham group, the expression levels of TNF-α, IL-1, and IL-10 in the spinal cord were significantly increased in the control group. The increase in TNF-α and IL-1 was significantly attenuated by TMP treatment ($P < 0.01$). Correspondingly, there was a significant elevation in the expression level of anti-inflammatory IL-10 in the TMP group ($P < 0.01$) [23].

4.9. Hydrogen. Hydrogen gas is a new popular therapeutic agent for tissue IRI. In a rabbit model of spinal cord IRI, the beneficial effects of hydrogen gas treatment against spinal cord IRI were associated with the decreased levels of proinflammatory cytokines (TNF-α) in serum and spinal cord [33].

4.10. Glycyrrhizin. Glycyrrhizin is a natural triterpene glycoconjugate derived from the root of licorice (Glycyrrhiza glabra). Glycyrrhizin might attenuate the transient spinal cord ischemic injury in rats via reducing inflammatory cytokines. In a rat model of spinal cord IRI, Glycyrrhizin reduced levels of TNF-α, IL-1β, and IL-6 in the plasma and injured spinal cord [34].

4.11. Panax Notoginsenoside. Panax notoginsenoside is an important traditional Chinese medicine and might exert potent neuroprotective effects on spinal cord IRI probably by its antiinflammation, antiedema, and antiapoptosis actions. In a rat model of spinal cord IRI, after Panax notoginsenoside or Methylprednisolone treatment, we observed reduced immunostaining of IL-1β, IL-10, and TNF-α, indicating that the infiltration of inflammatory cells was greatly relieved [18].

4.12. Intrathecal Transplantation of Bone Marrow Stromal Cells. In a rabbit model of spinal cord IRI, transplantation of bone marrow stromal cells reduced the excessive expression of TNF-α ($P < 0.01$) [35].

4.13. Ghrelin. In a rat model of spinal cord IRI, administration of ghrelin significantly attenuated the serum TNF-α level [24].

4.14. Thalidomide. Intraperitoneal (i.p.) administration of thalidomide might reduce IRI in a rabbit spinal cord model via reduction of TNF-α expression [25].

4.15. Antithrombin (AT). Antithrombin (AT) significantly inhibited the IR-induced increases in spinal cord tissue levels of TNF-α, rat IL-8, and myeloperoxidase [36].

4.16. Diltiazem. Diltiazem has cytoprotective and anti-inflammatory properties, leading to reduced spinal cord injury. In a rabbit model of spinal cord IRI (30-minute infrarenal aortic occlusion), diltiazem infusion significantly reduced IL-6 levels at 3 h and 24 h after reperfusion, and the mean IL-10 level in the diltiazem group was significantly higher than in the control group at 24 h after reperfusion [37].

4.17. Lazaroids. Lazaroids is 21-aminosteroids that might affect the production of both proinflammatory and anti-inflammatory cytokines in spinal cord IRI. In a rabbit model of spinal cord IRI (20-minute infrarenal aortic cross-clamping), plasma IL-8 and IL-1ra levels in lazaroids group were significantly lower than other groups ($P < 0.05$). Spinal IL-8 levels in lazaroids group (0.98 ± 0.34 ng/g tissue) were lower than those in control group (7.26 ± 2.26 ng/g tissue) ($P < 0.05$) [38].

4.18. Potential of Clinical Use. As reviewed in Section 3 "Cytokines in Spinal Cord IRI," inflammatory cytokines play an important role in spinal cord IRI as mediator. For treatment of several intractable autoimmune diseases (e.g., rheumatoid arthritis), where inflammatory cytokines are involved in disease progression, anti-inflammatory cytokine antagonist is now available. Hence, there is great potential of anti-inflammatory cytokine antagonist for therapeutic use of spinal cord IRI. Infliximab and other anti-TNF-α monoclonal antibodies are used mainly for treatment for autoimmune diseases [39]. The acute infusion reactions are well known as adverse effect of the monoclonal antibody therapy [40]. Therefore, the managing premedications for reactions to infusional monoclonal antibody therapy are mandatory [41, 42]. Among other inhibitors of inflammatory cytokines, IL-1ra is used to treat the symptoms of moderate to severe rheumatoid arthritis [43]. The most common side effect has included injection site reactions [44]. Besides the monoclonal antibodies of anti-inflammatory cytokines, Pentoxifylline, other TNF-α inhibitors, is already used clinically for treatment of intermittent claudication in certain patients to reduce pain, cramping, numbness, or weakness in the arms or legs and has been generally well tolerated. There are typically minor side effects for approximately 3% of treated patients with continuation of the drug.

Amongst more broad anti-inflammatory agents, ATL-146e is now in Phase III clinical trial as a pharmacological stress agent for use in myocardial perfusion imaging. ATL146e is more selective for A_{2A} receptor than CGS21680

and therapeutically more interesting, with lower side effects [45].

Instead of pharmaceutical drugs, the medical gases, nitric oxide, carbon monoxide, and hydrogen sulfide, have traditionally been considered to be toxic and environmentally hazardous, being now considerable therapy for spinal cord IRI. The numerous experimental animal and human studies of these agents have demonstrated protective effects against IRI. Effects of hydrogen have been reported in more than 60 disease models and human disease [46]. Only two diseases of cerebral infarction and metabolic syndrome have been analyzed in both animals and humans. It is noteworthy that, however, lack of any adverse effects of hydrogen enabled clinical studies even in the absence of animal studies.

5. Ineffective Treatments for Inflammation in Spinal Cord IRI

As researchers have attempted to discover more treatments for inflammation in spinal cord IRI, there are some strategies which were proved to be ineffective.

5.1. Heparin. The recently discovered anti-inflammatory property of glycosaminoglycans, including heparin, deserves to be investigated. However, in a rat model of spinal cord IRI, there was no significant difference between the groups in terms of the degree of inflammatory response, degree of IL-6, HSP-70, or MPO staining [47].

5.2. Carbamylated Erythropoietin Fusion Proteins or Recombinant Human Erythropoietin. In a swine model of spinal cord IRI, neither carbamylated erythropoietin fusion protein nor recombinant human erythropoietin affected the rise in IL-6 and TNF-α levels, and infiltration of inflammatory cells into the spinal cord did not show any intergroup difference [48].

6. Summary and Conclusion

Spinal cord IRI includes acute ischemic injury and delayed reperfusion injury. Inflammation is a subsequent event in both periods and a major contributor to spinal cord IRI. Inflammatory cells in spinal cord IRI mainly include macrophages, lymphocytes, neutrophils, microglia, and astrocytes. Inflammatory cells might participate in spinal cord IRI by inducing cell death and expressing inflammatory cytokines. Cytokines involved in spinal cord IRI include TNF-α, IL-1, IL-6, IL-8, and IL-10. Apart from IL-10, cytokines are characterized as proinflammation factors. The biphasic elevated expression of TNF-α, IL-1, and IL-8 might suggest a bimodal mechanism of spinal cord IRI. The effects of MEK/ERK pathway on inflammation might be mediated by inhibiting microglia activation and IL-1 production. Development and reactions of these inflammatory mediators should be fully elucidated. Diverse therapeutic strategies have been discovered for reducing inflammatory cells and cytokines in spinal cord IRI, including Adenosine A_{2A} receptor activation, inhibitor and antibody of TNF-α, IL-1 receptor antagonist and pathway inhibition, and other agents. The mechanisms and potency of these strategies deserve to be

further demonstrated, in order to provide safer and more effective treatments applied for clinical practice.

Conflict of Interests

The authors indicate no potential conflict of interests.

Authors' Contribution

Ping Zhu and Jia-xin Li have equal contributions to this work.

Acknowledgments

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Review Article

TLR2 and TLR4 in the Brain Injury Caused by Cerebral Ischemia and Reperfusion

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Brain injury caused by cerebral ischemia/reperfusion is a complicated pathophysiological course, in which inflammation is thought to play an important role. Toll-like receptors are a type of transmembrane proteins, which can recognize either exogenous pathogen-associated molecular patterns or endogenous stress or damage-associated molecular patterns in the innate immune system and initiate inflammatory responses. Among Toll-like receptors, TLR2 and TLR4 are found to be more important than others in the pathological progression of cerebral injury due to ischemia and reperfusion. This review will focus on the biological characteristics and functions of TLR2 and TLR4 and their downstream signal pathways.

1. Introduction

In 1980, a German biologist Nusslein-Volhard found a mutant gene in the embryos of Drosophila melanogaster, which was associated with regulating Drosophila dorsal-ventral polarity and was named "Toll gene" [1]. In 1984, Steward reported a "Toll protein" in Drosophila melanogaster, which was not only responsible for promoting dorsal-ventral differentiation, but could also recognize the invaded microbes and initiate antibacterial process [2]. In 1997, Janeway and Medzhitov reported firstly that a receptor, known currently as TLR4, was located on the human cell surface and was functionally similar to Drosophila Toll protein [3]. Thereafter, Medzhitov found some similar receptors in other animals or plants and named them Toll-like receptors (TLRs). By now, 10 functional TLRs in humans and 12 in rodents have been identified, respectively [4], of which TLR1-TLR9 were shared by both humans and rodents. Although it was initially thought that TLRs were associated with the development of mammals, Jules Hoffmann and Bruce Beutler found that TLRs are key sensors to infectious microorganisms in the mammalian innate immune response. Moreover, TLRs modulate the expression of inflammatory mediators when they were activated by host-derived molecules [5].

The mechanism underlying brain injury caused by cerebral ischemia and reperfusion remains elusive, but inflammatory response has been found to be one of the main factors leading to brain damage [6, 7]. Accumulating evidences show that TLRs (Toll-like receptors) are activated by endogenous proteins released from damaged brain and played crucial role in mediating the cerebral injury following ischemia and reperfusion [8]. In particular, TLR2 and TLR4 were found to be more important than other TLRs in the pathologic progression of cerebral ischemia and reperfusion [7, 9]. Therefore, TLRs have become a potential target in searching new strategy for the treatment of ischemic cerebrovascular disease. This review will focus on the biological characteristics and functions of TLR2 and TLR4 and their downstream signaling pathway.

2. Structure of Toll-Like Receptors

Toll-like receptors belong to the type I transmembrane glycoprotein receptor family. They are composed of extracellular N-terminal ligand-recognition domain, transmembrane domain, and intracellular C-terminal signaling domain [10]. The extracellular domain contains 16–28 extracellular leucine-rich repeat (LRR) modules and is mainly responsible

for binding to exogenous pathogen-associated molecular patterns (PAMPs) [11] and endogenous stress or damage-associated molecular patterns (DAMPs) [12]. The intracellular domain of TLRs is also known as Toll/IL-1 receptor (TIR) domain, because highly conserved sequences within it shared homologous to those of human cytoplasmic interleukin-1 receptor (IL-1R). Extracellular signals enter into cell and initiate the "signaling cascades" when the TIR domain was attached by intracellular specific adaptors [13], which include Myeloid differentiation factor 88 (MyD88), TIR domain-containing adaptor protein (TIRAP)/MyD88 adaptor-like (Mal), TIR domain-containing adaptor-inducing IFN-β (TRIF), TRIF-related adaptor molecule (TRAM), and Sterile α and armadillo motif-containing protein (SARM).

3. TLR2/TLR4 and Their Ligands in Cerebral Ischemic Reperfusion Injury

Toll-like receptors are found to present on various innate immune cells, such as polymorphonuclear neutrophils, monocyte/macrophage, dendritic cells, and NK cells, in which they trigger an immediate response against pathogens [14–16]. Within brain, they are mainly located on glial cells including microglia, astrocytes, and Oligodendrocytes. Microglia and astrocytes express a wide repertoire of TLRs [17], and they both could produce proinflammatory cytokines when TLRs are attached with their corresponding ligands [18–20]. By contrast, Oligodendrocytes can express a little repertoire of TLRs such as TLR2 and TLR3, which is mainly involved in central nervous system repair [21]. During recent years, the reports involving the expression of TLRs in neurons have sharply increased as well.

Although the central nervous system is a sterile circumstance and no pathogens such as germs or viruses exist under normal or ischemic condition, it is found that cerebral ischemia causes elevation in the expression of TLR2, TLR4, and TLR9 in neurons [22, 23]. By contrast, accumulating experimental evidences suggested that TLR2 and TLR4 play crucial roles in modulating inflammatory response caused by cerebral ischemia and reperfusion via linking to their endogenous ligands, respectively [24–26]. These endogenous ligands include heat-shock proteins (HSPs), high mobility group box 1 (HMGB1), hyaluronic acid, fibronectin [27, 28], and so on.

Heat shock proteins (HSPs) are normally in small quantity and function as chaperone to maintain effectively protein configuration. Heat shock protein family includes several members, such as HSP60, HSP70, and gp90. However, under the stress or damage conditions such as ischemia or hypoxia, their expression could be upregulated and they leak into the extracellular compartment to induce immune response and inflammatory response as endogenous ligands. During the course of cerebral ischemic/reperfusion injury, the binding of HSPs to TLR2 and TLR4 could initiate the signal pathway related to proinflammatory cytokines production [29]. Of all HSPs members, HSP60 and HSP70 are the most important endogenous ligands to TLR2 and TLR4. The binding of HSP60 with TLR2 and TLR4 leads to the recruitment of MyD88, activation of transcription factor, and increased expression of inflammatory cytokines TNF-α [30]. Similarly,

the binding of HSP70 with TLR2 and TLR4 activates the MyD88-IRAK-NF-κB signal pathways and promotes the transcription and expression of TNF-α, IL-1β, and IL-6 [31]. Brea et al. demonstrated in their vitro model study that blocking either HSP60 or TLR2 and TLR4 suppressed the strong inflammatory response in the cultures cells (monocytes and human umbilical vein endothelial cells) treated with serum from ischemic stroke patients [32]. This indicated that HSP60 might be a new therapeutic target for ischemic stroke.

High mobility group box 1 (HMGB1) belongs to the nuclear nonhistone DNA-binding protein family, which is responsible for maintaining the configuration of DNA in the nucleus and regulating gene transcription under normal conditions. HMGB1 participates in the inflammatory response under stress or damaging conditions when they leak into the extracellular compartment [33]. Peltz et al. found that plasma HMGB1 was markedly elevated within 2 to 6 hours in the acute damage event [34]. By using immunoprecipitation, researchers showed that HMGB1 can indeed be involved in the inflammatory responses through interacting with TLR2 and TLR4 [35]. The combination of HMGB1 with TLR2 or TLR4 activated its downstream MyD88-TIRAP-IRAK signal pathway to induce the release of cytokines such as TNF-α, iNOS, and ICAM-1. Moreover, the experimental data presented by Qiu et al. showed that the increased expression of MMP-9 in neurons and astrocytes following cerebral ischemia was triggered by HMGB1 predominantly via TLR4 pathway. Similarly, it was reported that the level of TLR4 increased in the primary cultured neurons and astrocytes treated with HMGB1. In the TLR2 overexpressional cells, HMGB1 can effectively induce the release of IL-8 and TNF-α. Thus, these studies showed that HMGB1 induce inflammatory response via TLR2 or TLR4 pathway. However, evidences from in vitro and in vivo studies showed that downregulating the expression of HMGB1 in neural cells was associated with the protection of short hairpin RNA (sh RNA) on ischemic neurodegeneration. It was also found that inhibition of HMGB1 expression reduced neuronal death, attenuated the activation of immune glia, and suppressed the induction of proinflammatory mediators such as iNOS, COX-2, IL-1β, and TNF-α in postischemic brain [36]. Particularly, in the case of transient middle cerebral artery occlusion (MCAO), the infarct volume was found to be markedly smaller in TLR4 mutant mice than that in the wild-type mice [37]. Anti-TLR2 antibodies diminished the expression of IL-8 and TNF-α via inhibiting the function of HMGB1 [38]. Therefore, as we discussed earlier, HMGB1 is an important ligand to bind with TLR2 and TLR4 and regulates the production of inflammatory factors.

Fibrin/fibrinogen is an acute reactive protein synthesized and secreted by hepatocytes and associated with coagulation process under normal condition. However, under stress or hypercoagulating state, fibrin/fibrinogen content will increase significantly and become a crucial factor in regulating the formation of thrombosis. Cerebral ischemic/reperfusion could induce accumulation of vascular fibrin/fibrinogen, which causes either the formation of microthrombosis resulting in microcirculation disorders, or upregulation in the expression of TLR4 and IRAK1 (interleukin-1 receptor
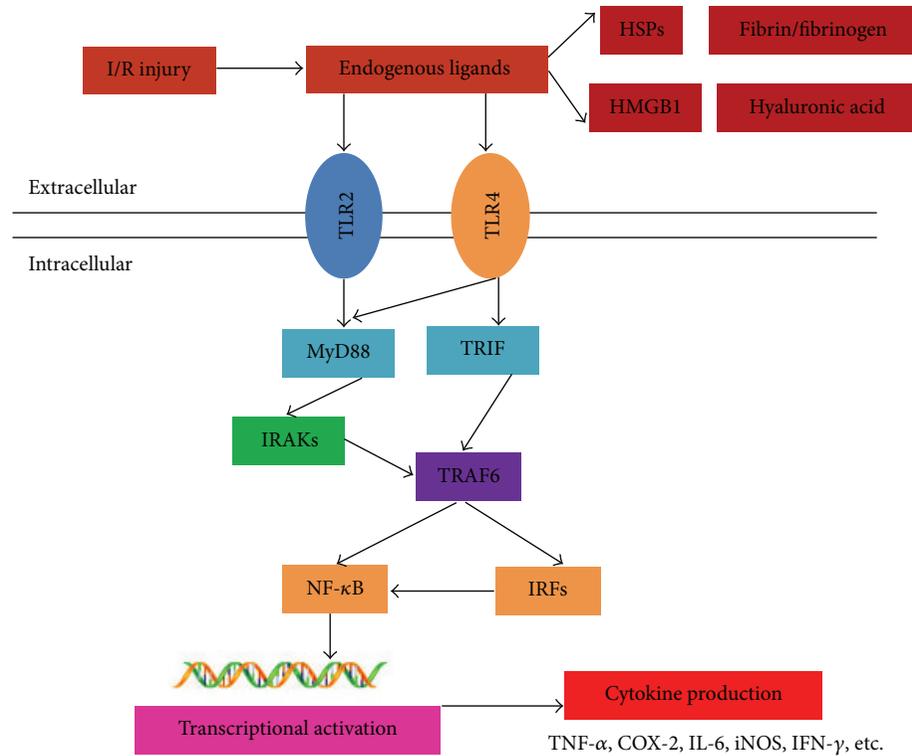
FIGURE 1: Brain injury caused by cerebral ischemia/reperfusion is a complicated pathophysiological course, in which TLR2 and TLR4 are thought to play an important role. TLR2 and TLR4 play crucial roles in modulating inflammatory response caused by cerebral ischemia and reperfusion via linking to their endogenous ligands and then recruit specific adaptors within cells. TLRs signal activates the transcription factors and generates cytokines and chemokines via intracellular pathways.

activated kinases 1) on the cerebral endothelial cells. Meanwhile, acute accumulation of fibrin/fibrinogen has been shown to induce proinflammatory responses as well as disruption in the blood-brain barrier via TLR signaling pathway during the course of cerebral ischemic insult [39, 40]. Zhang et al. demonstrated that the vessels with immunoreaction to fibrin/fibrinogen showed positive immunoresponses as well to TLR2, TLR4, and IRAK1, which suggested that the deposition of fibrin/fibrinogen on cerebral vessels induced by stroke could trigger the expression of TLRs. Combination therapy with VELCADE (a potent proteasome inhibitor) and tissue plasminogen activator (tPA) significantly reduced lesion volume and improved neurological score in MCAO rats. The authors thought that the therapy with VELCADE and tPA abolished the inactivation of fibrin/fibrinogen on NF-κB by directly inhibiting stroke-activated TLR2, TLR4, and IRAK1 and suppressing inflammatory respond [41].

Additionally, low molecular weight hyaluronan is also an endogenous ligand to TLR2 and TLR4. It activates the innate immune response via MyD88, IRAK, TNF-receptor association factor 6 (TRAF-6), and NF-κB pathway.

4. TLR2 and TLR4 Signaling

TLRs signal activates the transcription factors and generates cytokines and chemokines via intracellular pathways. TLR2

and TLR4 combined with their respective ligands to form dimeric complexes and change their configuration and then recruit five specific adaptors within cells including MyD88, TIRAP/Mal, TRIF, TRAM, and SARM.

TLR2 and TLR4 bind to these specific adaptors via their unique TIR domains. Depending on different adaptor proteins, the extracellular signals are transmitted to the downstream modules mainly via MyD88-dependent and MyD88-independent pathways (TIRAP/Mal, TRIF, TRAM, and SARM) (see Figure 1).

In MyD88-dependent pathway, MyD88 death domain interacted with interleukin-1 receptor-associated kinases (IRAKs) to activate TRAF-6. Following being phosphorylated by TRAF-6, the inhibitory κ kinases α/β (IκK α/β) activated transcription factor NF-κB and interferon promoter-binding protein (IRFs). Then, NF-κB and IRFs translocate into nucleus and induce the generation of a variety of cytokines, such as TNF-α, IL-1β, and IFN-β. TLR2 mainly transmits signals via MyD88-dependent pathway. In MyD88-independent pathway, TRIF interacts with TRIF-related adaptor molecule (TRAM), activates TRAF3, and phosphorylates IκKε. Finally, phosphorylated IκKε activates IRF3 and IRF7 and generates IFN-β. Besides MyD88 dependent and independent signal pathways, TLR4 mediates cell proliferation, transformation, and apoptosis via mitogen-activated protein kinases (MAPKs) signaling pathway [42].

5. TLR2 in Cerebral Ischemic/Reperfusion Injury

TLR2 is an important member in brain innate immune response system and mainly expressed on microglia, astrocyte, neuron, and endothelial cell. These glial cells could secrete the inflammatory factors, as well as generate proinflammatory and proapoptotic mediators related to TLR2 gene to exacerbate brain damage. To examine whether the TLR2 protein is of functional relevance to cerebral ischemia, brain damage was compared at reperfusion 2 days following 1 h MCAO in TLR2-deficient mice and wild-type mice. The result showed that the infarct volume in the TLR2-deficient mice was significantly smaller than that in the wild type mice [43]. Moreover, Lv et al. reported that sequential expressions of TLR2, IL-23, and IL-17 were observed either in microglia after cerebral ischemia/reperfusion or in cells cultured under the condition of oxygen-glucose deprivation reperfusion (OGDR) [44]. This study showed that microglia activated by ischemia/reperfusion or OGDR aggravated neuronal damage via secretion of toxic cytokines IL-23 and IL-17. By contrast, suppression of TLR2-IL-23-IL-17 axis in microglia leaded to reduction in neuronal apoptosis caused by OGDR or ischemia/reperfusion. These studies indicated that TLR2 plays a critical role in cerebral ischemic/reperfusion injury and initiates the inflammatory cascade to exacerbating brain damage. In the brain, the scavenger receptor CD36 is required for the inflammatory response triggered by TLR2 signaling. In the CD36 null mice, transient MCAO did not induce the expression of inflammatory genes thought to be upregulated by TLR2 and to aggravate brain damage. Thus, this indicated that TLR2-CD36 complex is a sensor of ischemia-induced prodeath signals and is critical for the inflammatory response [45]. Moreover, the same conclusion has achieved in accumulating experiments that TLR2 is a critical inflammatory trigger in cerebral ischemic/reperfusion injury [46, 47].

6. TLR4 in Cerebral Ischemic/Reperfusion Injury

TLR4 is a crucial receptor in the innate immune system and noninfectious immune responses. To clarify whether each of Toll-like receptors participate in the pathological course of cerebral ischemia/reperfusion injury, the size of brain infarction was compared by using TLR3, TLR4, TLR9 knock-out mice, and wild-type mice. The result showed that only the TLR4 knock-our mice has significantly smaller infarct volume at reperfusion 24 h after 2 h ischemia, when compared with wild-type mice [48]. This study suggested that TLR4 might play more important role than other Toll-like receptors during the course of brain damage caused by ischemia/reperfusion. The level of TLR4 mRNA increased in neurons after 1 h of cerebral ischemia, which was accompanied by the high level of multiple inflammatory cytokines [47]. Moreover, it was reported that LPS can serve as a potent preconditioning stimulus and provide protection against ischemic brain injury via modulating TRL4 [49]. Similarly, other experiments revealed that TLR4 could regulate the expression of cytokines, such as TNF-α, COX-2, IL-6, iNOS, and IFN-γ [50–53].

7. TLR2/TLR4 and Cytokines

Cerebral ischemic/reperfusion injury is accompanied with the inflammatory responses, which induce production of chemokines, adhesion molecules, and a large number of proinflammatory factors. IL-1β is an immune-derived cytokines and could be detected in brain at 30 min reperfusion after cerebral ischemia, which directly induces neuronal apoptosis and enhances the expression of chemokines within microglia and astrocyte. IL-1β possesses autocrine-like function and promotes the secretion of itself under ischemic stimuli. It is also considered to be a neurotoxic mediator, because infarct volume would decrease when its function is lost. In most cases, endogenous ligands binding to TLRs activate monocytes/macrophages to secrete IL-1β. Abulafia et al. reported that the level of IL-1βmRNA decreased in the TLR2 and TLR4 deficient mice which suffered from thromboembolic stroke [54].

TNF-α is another inflammatory cytokine participating in the pathological process of cerebral ischemia/reperfusion. Under normal circumstance, TNF-α is correlated with immune responses and could repair the cells of nervous system. It was found that the expression of TNF-α gradually increases in cerebral ischemia at 1 h reperfusion following cerebral ischemia. TNF-α promotes release of excitatory amino acid and production of oxygen free radicals. During the inflammatory procedure, TNF-α induces microglia and astrocyte to express cytotoxic iNOS. Meanwhile, the infiltration of leukocytes into brain parenchyma to aggravate ischemic injuries is also due to the assistance of TNF-α. Tu et al. used rat model of permanent MCAO to demonstrate that inhibition of TLR2 and TLR4 signaling pathway by baicalin reduced the volume of infarct brain and the serum level of TNF-α, IL-1β, and iNOS via inhibiting using [55].

IL-6 possesses multiple biological functions and has been mainly secreted by astrocyte and microglia during the course of cerebral ischemia/reperfusion. Moreover, a variety of cytokines can induce the production of IL-6 as brain tissue suffered from the damage stimulus. IL-6 can be detected in the brain tissue, cerebrospinal fluid, and serum at reperfusion 2 h after cerebral ischemia. However, some studies have shown that IL-6 could stimulate astrocyte to generate neurotrophic factors and nervous growth factors, which assist the repair of damaged nervous cells via JAK or STAT pathways but also participate in the inflammatory injuries as well [56]. Cao et al. demonstrated, by using mice model of MCAO, that the neurological impairments and the level of IL-6 were both improved more obviously in TLR4 deficient mice than in wild-type mice [51].

Chemokine is a family of small cytokines and can enhance inflammatory response. Meanwhile, chemokine could act as a chemoattractant to guide the migration of cells and promote leukocytes infiltrating into brain tissue during the process of cerebral ischemia and reperfusion. It was found that the proliferation capacity of resident microglia and the level of monocyte chemotactic protein-1 (MCP-1) were both

reduced in the TLR2 deficient mice treated with transient MCAO, when compared with that in the wild-type mice [57]. Adhesion molecules are located on cell surface to assist cells sticking to each other and their surroundings. For an instance, adhesion molecule ICAM could induce leukocyte rolling and sticking to the vascular endothelial cells, which lead to the penetration of leukocyte into extravascular matrix to release cytotoxic protease and aggravation of brain injuries caused by cerebral ischemia/reperfusion. In vitro, treatment with rhHMGB1 made the expression of TNF-α and ICAM-1 up-regulated in the primary cultured neurons and endothelial cells that expressed TLR2 and TLR4 [58]. Again, TLR2 and TLR4 binding to HMGB-1 indeed promotes inflammatory responses.

8. Inference

There is a definite conclusion on the basis of current findings that TLR2 and TLR4 exert key influences on the pathological process of cerebral ischemia/reperfusion. The common characteristics are that (1) the expressions of TLR2 and TLR4 increase at the beginning of reperfusion following cerebral ischemia and last a long time; (2) the methods inhibiting the expression of TLR2 and TLR4 in brain tissue have significantly suppressed neurological deficits and alleviated brain damage caused by cerebral ischemia and reperfusion; (3) the expressional level of TLR2 and TLR4 influenced the production of multiple cytokines which participate in inflammatory signal pathway and decided the outcome of cerebral ischemia and reperfusion. Clinical study showed that the level of TLR2 and TLR4 in neutrophils at 72 h and 7 days following ischemia and reperfusion was an independent indicator associated with the volume of brain infarction and prognosis of stroke patients [59]. Moreover, it was found that the level of TLR4 mRNA elevated significantly within peripheral blood in patients with transient ischemic attack (TIA) or stroke when compared with that in the patient with asymptomatic internal carotid artery stenosis [60]. Therefore, inhibition of TLR2 and TLR4 has become a strategy to prevent or treat ischemic cerebrovascular diseases.

9. Prospective

Some compounds or chemicals such as baicalin, luteolin, and picroside II have been selected to inhibit TLR2 and TLR4. Neurological deficit, infarct volume, the expression of TLR2, TLR4, and a variety of inflammatory cytokines in the damaged brain tissue were examined at different reperfusion time points by using rat MCAO model. The results showed that most of previously mentioned compounds could alleviate brain damage caused by cerebral ischemia/reperfusion via inhibiting the expression of TLR2, TLR4, and inflammatory cytokines NF-κB, iNOS, COX-2, IL-6, and TNF-α [55, 61, 62].

By contrast, some ligands of TLR4 or TLR2 could be used as preconditioning inducer, which would enhance cerebral resistance to severe ischemia and reperfusion. The mice pretreated at 72 h prior to cerebral ischemia and reperfusion with subcutaneous injection of low dose of LPS, a specific ligand

of TLR4, showed decreased brain infarction. The possible mechanisms are considered to be via suppressing TLR4-related inflammatory signals such as MyD88-TRIF-IRF3-NF-κB pathway [63], or inducing production of neuroprotective cytokines TGF-β and IL-10. Similarly, preconditioning with TLR2 specific ligand, Pam$_3$CSK$_4$, could significantly reduce neuronal apoptosis and brain damage caused by cerebral ischemia/reperfusion via activation of TLR2-PI3K-Akt pathway [64].

Experimental and clinical studies have demonstrated that physical exercise benefits neurological recovery or reduction of neurological dysfunction caused by cerebral ischemia and reperfusion. Mild treadmill training at three weeks prior to brain ischemia, or at day 5 reperfusion following ischemia, contributed to the recovery of neurological dysfunction via inhibiting the mRNA level and protein concentration of TLR2 and TLR4 and their downstream molecules (such as MyD88 and NF-κB) in rat brain tissue [65, 66].

Acupuncture is an important procedure in traditional Chinese medicine and has been used as a complementary and alternative treatment for neurological recovery. Electric stimulation to some acupoints (such as Quchi and Zusanli) has been demonstrated to be neuroprotective and anti-inflammatory. Further study showed that the effects of electroacupuncture on improving significantly the neurological deficits, reducing infarct brain tissue, and alleviating inflammatory responses were via inhibiting TLR4 signaling pathway [67].

Moreover, other means have also been exerted to suppress overexpression of TLR2 and TLR4 during cerebral ischemia/reperfusion. As mentioned earlier, CD14 is an important molecule to assist the formation of TLR4 homodimer. Canavanine, an iNOS selective inhibitor, not only inhibited the elevation of CD14 and TNF-α in microglia exposed to hypoxia circumstance, but also suppressed TLR4 to form homodimer and blocked its downstream signaling pathways [68].

10. Conclusions

Brain injury caused by cerebral ischemia/reperfusion is a complicated pathological course, in which inflammatory response plays a crucial role. Moreover, accumulating evidences showed that TLR2 and TLR4 are the most important Toll-like receptors during this process. A variety of means have been used to demonstrated the effectiveness of inhibiting the expression of TLR2 and TLR4 and their downstream signaling pathways on protecting brain injury caused by cerebral ischemia and reperfusion. However, further studies are also needed to screen out a medicine targeting to block TLR2 and TLR4 and could be used in future clinical practice.

Acknowledgment

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Review Article

Role of Regulatory T Cells in Pathogenesis and Biological Therapy of Multiple Sclerosis

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Multiple sclerosis (MS) is an inflammatory disease in which the myelin sheaths around the axons of the brain and spinal cord are damaged, leading to demyelination and scarring as well as a broad spectrum of signs and symptoms. It is caused by an autoimmune response to self-antigens in a genetically susceptible individual induced by unknown environmental factors. Principal cells of the immune system that drive the immunopathological processes are T cells, especially of T_H1 and T_H17 subsets. However, in recent years, it was disclosed that regulatory T cells took part in, too. Subsequently, there was endeavour to develop ways how to re-establish their physiological functions. In this review, we describe known mechanisms of action, efficacy, and side-effects of contemporary and emerging MS immunotherapeutical agents on Treg cells and other cells of the immune system involved in the immunopathogenesis of the disease. Furthermore, we discuss how laboratory immunology can offer physicians its help in the diagnosis process and decisions what kind of biological therapy should be used.

1. Introduction

The physiological function of the immune system is defence against external and internal violators of integrity of the organism. External "enemies" are represented mainly by germs; those of internal origin belong especially to potentially malignant cells that appear in our organisms as a result of the breakdown of their replication mechanisms. Another important biological function of the immune system is the prevention of autoreactive T and B cells activation, respectively, which potentially represent a threat of autoimmune diseases induction. To avoid this possibility, mechanisms of recessive (central) and dominant (peripheral) tolerance were developed. Recessive tolerance is based on deletion of autoreactive T and B cells in the thymus or in the bone marrow, respectively, during the process of their maturation in these primary lymphoid organs [1, 2]. Like other biological systems, the mechanisms of the recessive tolerance are not 100% effective, and a part of autoreactive lymphocytes escape their demise and enter the periphery, the secondary lymphoid organs. Here, when they encounter autoantigens, cross-reactive antigens or when a dysregulation of the immune system develops,

they can be activated and induce autoimmune processes. Mechanisms of dominant tolerance mediated mainly by regulatory T cells (Treg) prevent this eventuality. By contacting with autoreactive lymphocytes directly or indirectly, especially by synthesis of immunosuppressive cytokines, Treg cells prevent their activation or suppress their effector activity [1, 2].

2. Regulatory T and B Cells

Regulatory T cells are divided into two populations: natural and induced (adaptive). Natural Treg cells (nTreg) represent an independent population, such as B lymphocytes, NK, and NKT cells. On the other hand, induced regulatory T cells (iTreg) is a population that develops during the immune response only; they represent a subset of $CD4^+$ T helper cells [3, 4].

Natural regulatory T cells differentiate in the thymus. To develop, their T cell receptor (TCR$\alpha\beta$) must recognise peptides originating from self-antigens presented by HLA molecules in membranes of dendritic cells (DC); the recognition is

highly affinitive. Moreover, costimulatory interactions between CD28 (nTreg) and CD80/CD86 (DC) as well as signalling processes induced by IL-2 or IL-15 are indispensable for their differentiation [3, 5].

Signalling processes result in formation of various transcription factors such as BLIMP-1, IRF4, transcription factors of the FOXO family, STAT5, and especially FOXP3 (forkhead box P3). It binds DNA and acts as a transcriptional activator/repressor by recruiting deacetylases as well as histone acetyltransferases. FOXP3 is crucial for the function of the nTreg cells. Humans with mutations in the FOXP3 gene (Xp11.23-q13.3) suffer from a severe autoimmune disorder known as IPEX syndrome (immune dysregulation, polyendocrinopathy, enteropathy, and X-linked), which manifests in lymphoproliferation, multiorgan lymphocytic infiltration, and systemic autoimmune inflammation; the disease is incompatible with life [6, 7].

nTreg cells comprise 5–10% of all CD4$^+$CD8$^-$ thymocytes and in the periphery they represent approximately 10% out of the whole population of CD4$^+$ T cells. nTreg cells are long-living cells and IL-2 is essential for their peripheral maintenance as proved by its neutralisation, which results in reduction of nTreg cells counts and sensitisation to autoimmunity [8–10]. They are characterised by several membrane molecules. Characteristically, they express CD4, CD25, CD5, CD49d, CD69, CD103, CD152 (CTLA-4), and CD357 (GITR). More typical signs are low expression of CD127 (alpha chain of IL-7 receptor) and high expression of neuropilin [3, 11].

nTreg cells are anergic, that is, they do not respond to *in vitro* stimulation by anti-CD3 monoclonal antibodies, phytohaemagglutinin, or allogeneic cells and do not produce IL-2. However, they downregulate activities of T cells, B, NK, NKT, and dendritic cells. The mechanisms they use include the production of immunosuppressive cytokines (IL-10, TGF-β, and IL-35), induction of apoptotic processes and metabolic alterations in target cells, and interference with maturation of dendritic cells [12–14].

Induced regulatory T cells differentiate from naïve CD4$^+$ T helper cells during the immune response. They develop especially in a mucosal environment; their differentiation is best elucidated in the intestine. iTreg cells appear after naïve CD4$^+$ T-lymphocytes are exposed to antigens, TGF-β, IL-2, and retinoic acid produced by DCs from vitamin A [2, 4, 15]. iTreg cells differentiate under suboptimal TCR stimulation without the necessity of CD28 costimulation. Like the nTreg cells, they also need FOXP3 for their development; however, a different part of proximal conserved noncoding sequences in its locus (CNS1) and different transcription factors (esp. NFAT). No systemic disease similar to IPEX happens when there is a deficiency of iTreg cells (in experimental animals); however, inflammatory processes of mucosal surfaces develop (colitis, asthma bronchiale) [16–18]. Initially, iTreg and nTreg cells were difficult to distinguish from one another. The problem was resolved only recently when it was found that iTreg, unlike nTreg, expressed much lower quantities of neuropilin [19]. iTreg cells suppress activities of target cells in a similar way like nTreg cells [4, 15].

What is the basic difference between nTreg and iTreg cells? Both suppress activities of cells of the immune system.

nTreg cells principally downregulate activities of autoreactive T cells, that is, those that escaped from their demise in the thymus, for example, those that recognise myelin basic protein (MBP). iTreg cells restrict activities of effector T cells induced during the immune response to antigens, for example, the ones that are present in our intestines as commensals. nTreg and iTreg cells are not interchangeable in their activities; they complement each other [16].

B cells, to our surprise, can act as immunosuppressive cells too. Their downregulatory activity is mediated either by a direct contact with targets cells or by TGF-β, and especially by IL-10, or they can induce apoptosis of activated T cells. Their characteristic membrane molecules are known in mice, not yet in humans. They are designated as Breg or B10 cells [20–22].

3. Immunopathogenesis of Multiple Sclerosis and Neuromyelitis Optica

Multiple sclerosis (MS) is a characteristic autoimmune disease. In short, MS is characterised by demyelination process in the brain and the spinal cord; the peripheral nervous system is rarely involved. Aetiology of the disease is still unknown, most likely MS occurs because of some combination of genetic, environmental, and infectious agents, among them EBV, CMV, HBV, HSV, human herpetic viruses 6, or 7, measles viruses, coronaviruses, and others. A relationship between viruses and MS is supported by observations that viral infections frequently precede bouts of the disease. It is possible that IFN-γ, which is produced during the infection, triggers immunopathological events resulting in demyelination [23]. The viruses may possess proteins that resemble the myelin antigens and by the mechanisms of molecular mimicry activate autoreactive T cells. Environmental factors like insufficient supply of vitamin D seem to support MS development [24, 25]. Furthermore, MS prevalence rates may be influenced by the socioeconomic changes in previous decades, which are related to industrialisation, urban living, pollution, occupational exposures to solvents, changes in diet, smoking habits, and so forth.

Immunopathological processes start by activation of autoreactive T cells in the periphery; they belong to T_H1, and to T_H17 subsets (Figure 1) [23, 26]. Activated T cells subsequently upregulate the expression of adhesive molecules which enables them to adhere to their counterparts in membranes of endothelial cells of the central nervous system (CNS). LFA-1 and $\alpha4\beta1$-integrins in T cells and ICAM-1 and VCAM-1 in endothelial cells mediate these interactions. VCAM-1 is constitutively expressed and its expression is substantially upregulated by stimulation of cells by cytokines. This mechanism of transmigration into the CNS parenchyma is used mainly by T_H1 cells. T_H17 cells prefer interaction between their chemokine receptors CCR6 and CCL20 ligands, which are also constitutively expressed in small quantities in membranes of endothelial cells [27, 28]. Likewise, B cells migration across the blood-brain barrier (BBB) is mediated by interaction between their CCR7 and CCL19 in the brain [28].
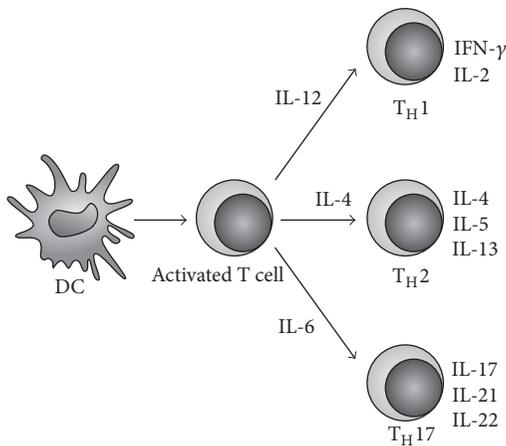
FIGURE 1: Differentiation of naïve T helper cells into particular subsets. T helper lymphocytes leaving the thymus (naïve or T_H0) are not yet fully differentiated to perform their specific functions in peripheral lymphoid tissues. They are endowed of these properties in the process of their interactions with dendritic cells (DCs) that engulf, process, and present antigens to them. Moreover, DCs in dependence of the processed antigens produce different cytokines. If DCs produce IL-12, naïve T cells polarise into the T_H1 subset, if IL-4 into the T_H2 subset and eventually, if DCs synthesise IL-6, naïve T helper cells will become the T_H17 cells.


FIGURE 2: Causes of impaired Treg cells function in autoimmunity development. Failures of regulatory T (Treg) cell-mediated regulation can include: inadequate numbers of Treg cells owing to their inadequate development in the thymus, for example, due to a shortage of principal cytokines (IL-2, TGF-β) or costimulatory signals (CD28), and so forth. Further, the number of Treg cells can be in a physiological range; however, there are some defects in Treg-cell function that are intrinsic to Treg cells, for example, they do not synthesise sufficient quantity of immunosuppressive cytokines (IL-10, IL-35, and TGF-β), or there is a breakdown of their interaction with effector T cells. Ultimately, pathogenic effector T cells (Teff) are resistant to suppression by Treg cells owing to factors that are intrinsic to the effector cells or factors that are present in the inflammatory milieu that supports effector T cells resistance.

Upon entering the CNS, T cells are reactivated by local and infiltrating antigen presenting cells (i.e. dendritic cells and macrophages), which present peptides originated from myelin by their HLA class II molecules to T cells [29–31]. The activated T cells migrate into the parenchyma and produce proinflammatory cytokines (esp. IFN-γ, IL-17), which themselves may damage myelin [26]. However, more importantly, they activate microglial cells, which are thought to be the main cells responsible for lesional and perilesional axon killing. They, by the synthesis of cytokines (IL-12, IL-23, osteopontin), reactive oxygen, and nitrogen intermediate products, further contribute to the damage of myelin sheaths resulting in impaired nerve conduction [26, 30].

Studies on experimental autoimmune encephalomyelitis (EAE) induction suggested that only T_H1 cells could access the CNS initially and this facilitated subsequent recruitment of T_H17 cells [32]. They produced various cytokines, especially IL-17 and they themselves killed human neurons and promoted central nervous system inflammation through $CD4^+$ lymphocyte recruitment [23, 33, 34]. However, perhaps more important than IL-17 production is their synthesis of GM-CSF. This cytokine supports attraction of monocytes and dendritic cells to the CNS and their activation, the mechanisms by which they contribute to neuroinflammation. GM-CSF furthermore acts as a positive feedback loop whereby it enhances the synthesis of IL-23 from antigen-presenting cells and so further sustains the activation and maturation of T_H17 cells [35].

MS has been viewed historically as a $CD4^+$ T cell-mediated autoimmune disease. However, the frequency of $CD8^+$ T cells is greater than that of T helper cells in inflamed plaques, and $CD8^+$ T cells show oligoclonal expansion in plaques, CSF, and blood which suggests they have a pathogenic role in MS too. Cytotoxic T cells destroy myelin by their perforin-granzyme mechanisms, resulting in the release of other autoantigens and circulus vitiosus continues [23, 36].

Regulatory T cells play a vital role in the regulation of immune processes. Based on the induction of autoimmune processes caused by the FOXP3 gene mutation, it was supposed that defective Treg cells might also contribute to the development of immunopathological processes in "more common" autoimmune disorders. This supposition has been confirmed. nTreg cells can contribute to the induction of autoimmunity by their decreased numbers, by the breakdown of their function, or simply by the reality that overactivated autoreactive T cells resist their immunosuppressive activities (Figure 2). The role of Treg cells in MS is rather controversial. While there have been reports on reduced frequency of nTreg cells in MS patients [37, 38], the majority of studies have found a similar frequency to the one observed in healthy individuals [39, 40]. However, several functional studies using *in vitro* suppression assays have documented impairments in Treg cells from MS patients [40–43]. What may be the cause for insufficient activities of nTreg cells in MS patients? It is probably a complex defect, such as reduced expression of coinhibitory molecules (CLTA-4, TIM-3, TIGIT) in their membranes, and insufficient synthesis of immunosuppressive cytokines [42, 44]. In this context, an interesting finding was reported by Schneider-Hohendorf et al. They disclosed an impaired migratory activity of Treg cells into the CNS in patients with relapsing-remitting MS (RR-MS) [45].

Adoptive transfer and depletion experiments in mice have also provided evidence that Treg can control the development and severity of EAE. For instance, in MOG-induced EAE, the transfer of Treg cells reduced disease severity and they were also able to suppress MOG-specific T cell responses *in vitro* [46]. In another study, in the PLP-induced model, the susceptibility of different mouse strains to EAE correlates inversely with the frequency of PLP-specific Treg cells [47]. These studies and others suggest that Treg cells influence the susceptibility to development of disease in the EAE models.

B cells do not cross the intact blood-brain barrier. However, once inflammation has started, they can enter the CNS. B cells, plasma cells, and myelin-specific antibodies are detected in MS plaques and in areas of active demyelination in MS patients [48, 49]. Recent studies have identified ectopic lymphoid follicles resembling germinal centres in the meninges [50–52]. It is possible that clonally expanded B cells, which originated in the meninges, may migrate to the parenchyma and participate in CNS damage. However, others did not confirm the findings [53], so the role of follicles remains controversial for the moment.

The intracerebral synthesis of IgG is typically oligoclonal; exact target antigens of these antibodies are, however, still elusive [23, 54, 55]. Antibodies can cause demyelination by opsonisation of myelin for phagocytosis or via complement activation. Besides the antibodies production, B cells have several antibody-independent functions. They include antigen presentation, T cell activation, and production of effector cytokines as reflected by introduction of anti-CD20 monoclonal antibodies to the treatment of MS patients (see later).

Ultimately, over previous decenniums, several authors have found NK cell defects in MS. It is, however, not yet known whether they are responsible for the development of the disease or only secondarily reflect the ongoing immunopathological process [35].

Until recently, neuromyelitis optica (NMO), also known as Devic's disease or Devic's syndrome, was considered a variation of multiple sclerosis. Now, it represents an independent disease, in which a person's own immune system attacks the optic nerves and spinal cord [32]. Although inflammation may also affect the brain, the lesions are different from those observed in MS. Unlike standard MS, the attacks are not mediated by the immune system's T cells, but rather by antibodies directed against aquaporin 4 (AQP4), a protein in the cell membranes of astrocytes. However, as antibodies belong to IgG1 class, their production requires T cells [56, 57]. Moreover, Varrin-Doyer et al. have brought evidence that T cells from NMO patients proliferated to intact AQP4 or AQP4 peptides [56].

Aquaporin 4 acts as a channel for transporting water across the cell membrane [58, 59]. In the processes of astrocytes that surround the BBB, a system responsible for preventing substances in the blood from crossing into the brain is found. It is currently unknown how the antibodies lead to demyelination. However, the induction of NMO seems to be resolved. Recently, some papers were published indicating the existence of structural homology and cross-reactivity between water channel proteins of *Helicobacter pylori* [60], *E. coli* aquaporin Z [61], and *Clostridium perfringens* adenosine

triphosphate-binding cassette (ABC) transporter permease [56] and aquaporin 4, respectively.

Dominant cells that infiltrate the NMO lesions are neutrophils, the cells practically absent from lesions in MS. Their recruitment and activation can be mediated by IL-6, IL-8, and G-CSF. Levels of these cytokines were elevated in the cerebrospinal fluid (CSF) [62] as well as those of IL-17 [63].

Interferon beta (IFN-β), which has been used in the treatment of MS, must not be prescribed for the NMO treatment. Not only do patients exist who do not respond to the treatment, but moreover, IFN-β induces severe relapses and exacerbations of the disease in some of them [64, 65]. There is no cure for NMO. Currently azathioprine, prednisone, rituximab, cyclophosphamide, methotrexate, mitoxantrone, mycophenolate mofetil, intravenous immunoglobulins, or exchange plasmapheresis have been used for the treatment. However, recently it was shown that biological agents might be of some benefit in ameliorating a clinical status of the patients. New monoclonal antibodies, aquapuromab, were developed which also bind to AQP4, however to different epitopes as autoantibodies. Their attachment to AQP4 prevents pathogenic autoantibodies to bind to their targets because of steric hindrance and so to prevent their pathogenic activities. Aquaporumab activates neither the complement system, nor killer (K) cells, which prevents potential damage of target cells they bind with [66].

4. Biological Therapy of Multiple Sclerosis

A better understanding of the underlying mechanisms of MS found its reflection in the development of various immunotherapeutic agents. The first biologic agent used in the treatment of MS was IFN-β (1993), followed by glatiramer-acetate, monoclonal antibodies, FTY-720, and others. Each of them influences the ongoing immunopathogenic processes differently, trying to reestablish a previous physiological state (Table 1). However, it must be stressed that none of them has achieved its goal; all ameliorated the clinical state of treated patients only; no one was cured.

4.1. The First Line Agents for the Treatment of MS. Interferon beta is a cytokine with more immunomodulatory properties. It downregulates the expression of HLA class II molecules in antigen-presenting cells (APCs), which results in decreasing peptide presentation to T cells. On the contrary, it upregulates the expression of PDL-2 inhibitory molecules, which when interact with PD1 receptors in membranes of T cells, induce their apoptosis. IFN-β also inhibits proliferation of macrophages, resulting in reduction of their numbers and so activation of autoreactive T cells. Furthermore, IFN-β decreases also the transmigration of activated T cells into the CNS by the downregulation of their VLA-4 adhesive molecules, which are vital for binding to their VCAM-1 partners in membranes of endothelial cells [67].

IFN-β influences also activities of Treg cells. It upregulates the number of ligands for GITR receptors in membranes of dendritic cells. The interaction between GITR in Treg-cells and GITR-ligands in dendritic cells induces the proliferation

Table 1: Mechanisms of action of contemporary and emerging MS therapies.

Drug	Mechanism of action
IFN-β and Glatiramer acetate	Inhibition of the induction and proliferation of autoreactive T cells
Fingolimod	Prevention of egress of CD4$^+$ & CD8$^+$ T cells, and B cells from secondary lymphoid tissues
Natalizumab	Blockade of transmigration of autoreactive T cells into the CNS
Rituximab	Depletion of B cells and attenuation of antibody independent proinflammatory B cell functions
Alemtuzumab	Depletion of CD4$^+$ & CD8$^+$ T cells, B cells, NK cells, and monocytes
Daclizumab	Expansion in CD56^{bright} NK cells; inhibition of activated T-cell proliferation

of Treg lymphocytes, followed by an increase of their numbers and more active suppressive activities. The proliferation of Treg cells is further supported by other effect of IFN-β; it also downregulates the number of CTLA-4 molecules, which inhibit activities of Treg cells. This way, they become more susceptible for stimulatory cytokines, especially of IL-2, which is their basic homeostatic cytokine [68]. These experimental findings are corroborated by the results of the IFN-β treatment of MS patients with impaired nTreg function, which was shown to be reversed [41, 69].

Glatiramer acetate (GA) belongs to the first lineage of drugs used to treat MS. It is a random polymer of four amino acids found in myelin basic protein, namely, L-glutamic acid, L-lysine, L-alanine, and L-tyrosine. The mechanism of GA activity might be based on a blockade of grooves of HLA molecules. However, it seems that GA is endowed by immunomodulatory properties too. It was proved to induce, like IFN-β, the production of IL-1Ra, a natural inhibitor of IL-1, which results in inhibition of its proinflammatory activities. Furthermore, monocytes/macrophages under the GA activities produce less IL-1 and TNF, that is, the most potent proinflammatory cytokines and IL-12, the cytokine supporting polarisation of naïve T cells into the T$_\text{H}$1 subset (Figure 1). On the contrary, it increases the synthesis of immunosuppressive IL-10 [70]. One supposes that GA-activated T cells enter the CNS and develop their anti-inflammatory and neuroprotective activities [71]. GA supports also suppressive activities of Treg cells by the upregulation of their coinhibitory molecules TIGIT and TIM-3 [42, 44, 72]. Like the treatment with IFN-β, that with GA resulted in reversal of impaired nTreg cells function [73].

4.2. Second-Line Agents for MS Treatment.
Patients who are suboptimal responders to the standard immunomodulatory therapies are considered for treatment with second-line therapy represented by natalizumab and FTY720.

Natalizumab is a humanised monoclonal antibody against the cell adhesion VLA-4 molecule, its alpha 4 chain (VLA-4 belongs to beta-1 integrins: α4/β1). VLA-4 is located in

membranes of T cells and its partner molecule is VCAM-1 in membranes of cerebral endothelial cells. This way, natalizumab prevents a transmigration of activated T cells into the CNS because they do not succeed in adhering to endothelial cells; macroscopically, this effect of natalizumab is perceived as lymphocytosis. Regulatory T cells are not affected by natalizumab; it influences neither their number nor function. Natalizumab reduces also the number of dendritic cells in the perivascular environment of the brain, indicating that its activity does not restrict itself to T cells only [74].

For some patients, discontinuation of the natalizumab treatment results in disease reactivation. Subjects who relapsed or had magnetic resonance imaging (MRI) worsening after treatment cessation had milder peripheral lymphocyte increases during the treatment. Furthermore, patients carrying a variant of the gene coding for Akt associated with reduced antiapoptotic efficiency (rs2498804T) had lower lymphocytosis and higher risk of disease reactivation [75, 76].

Natalizumab therapy may be associated with progressive multifocal leukoencephalopathy (PML), a potential life-threatening complication. PML is thought to be caused by reactivation of John Cunningham virus (JCV), primarily in the setting of immunosuppression [77]. Its pathological activity results in oligodendrocyte destruction [78]. An other complication of the natalizumab treatment is the induction of IRIS (immune reconstitution inflammatory syndrome), also known as the "immune recovery syndrome." It is observed in some patients recovering from immunosuppression in whom the immune system begins to revive but then responds to a previously acquired opportunistic infections with an overwhelming inflammatory response that paradoxically makes the symptoms of the infection worse [78, 79]. Treg cells, rather induced than natural, probably take part in IRIS induction too because of inappropriate conditions for their induction. Nevertheless, natalizumab holds its position in the MS treatment when a physician considers the risk of the patient to develop PML or IRIS, and when his/her previous immunosuppressive treatment and a positivity of anti-JVC antibodies are taken into account.

FTY720 (fingolimod), a derivative of myriocin, a fungal metabolite of the Chinese herb *Iscaria sinclarii*, is an other second-line immunomodulating drug approved for treating MS. It is a structural analogue of intracellular sphingosine that is phosphorylated by sphingosine kinase 2 *in vivo*. Fingolimod exerts its effect by mimicking sphingosine 1-phosphate (S1P) and the binding to four of five S1P receptors on lymphocytes results in their internalisation and prolonged downregulation. Without signals from S1P receptors, CD4$^+$ and CD8$^+$ T cells and B cells are unable to egress from secondary lymphoid tissues, resulting in a marked decrease of these cells in the periphery and their reduced recruitment to sites of inflammation. Approximately 80% of lymphocytes undergoes this reversible sequestration 3–5 hours after fingolimod application [80, 81].

Data on fingolimod effect on regulatory T cells are contradictory. There are reports claiming that it supports their proliferation and immunosuppressive activities although the mechanisms by which it exerts these effects are not reported [80]. The positive influence of fingolimod on Treg cells seems

to be supported by clinical experience as its discontinuation in the treatment can result in relapse and induction of symptoms resembling IRIS [82, 83]. It can indicate that cessation of fingolimod treatment resulted also in reduction of Treg cells immunosuppressive activities and subsequently in reactivation of effector T cells.

On the other site, there are also reports showing fingolimod decreases activities of Treg cells. The way how fingolimod downregulates a Treg immunosuppressive potential is based on blocking IL-2-induced expansion, which is indispensable for their *in vivo* immunosuppressive activity [8, 84]. However, clinical experience connected with the above-mentioned relapse of the disease after fingolimod discontinuation does not support these results, or the results obtained in preclinical experiments do not always need to correlate with those when drugs are used in the real treatment of patients. It reminds of the events from 2006 when the superagonistic monoclonal antibodies anti-CD28 were applied to 4 volunteers. The antibodies supported the expansion of Treg cells in preclinical testing with mice; however, with the volunteers, they induced a cytokine storm and severe clinical symptoms threatening their lives [85].

4.3. Emerging Biological Agents for MS Treatment. The last decennium has brought the development of new biological agents that can modulate the MS disease processes, and we are now witnesses of many trials to verify their modes of action, benefits, and adverse reactions. Among them are novel monoclonal antibodies (mAb), especially anti-CD20, anti-CD52, and anti-CD25.

Anti-CD20 monoclonal antibodies bind to B cells and by activation of the complement system or killer cells, they destroy them. The rationale of a decrease of B cells for MS (and other autoimmune disorders) treatment is based on their other functions, not only those connected with production of antibodies. B cells belong to antigen-presenting cells too. They express HLA class II molecules, and engulfed protein antigens, previously bound to their immunoglobulin receptors, are then subsequently processed and bound to their grooves. The presentation of the "HLA-molecule—peptide" complex to T cells follows and by receiving costimulatory signals, T cells are activated [86, 87]. By destruction of B lymphocytes, anti-CD20 mAb reduce their number and so downregulate their ability to interact with autoreactive T cells, which results in attenuation of autoimmune processes. Concurrently, a cytokine profile in the microenvironment is changing in support of the induction and expansion of Treg cells [20, 88, 89]. Why was the CD20 molecule selected? The answer is relatively easy: CD20 is expressed on B cell lineage from the pre-B cell stage to the memory B cell stage, but not on plasma cells [90].

There are three different types of anti-CD20 mAb: rituximab, ocrelizumab, and ofatimumab. Rituximab and ofatumumab destroy B lymphocytes by the complement system activation, whereas ocrelizumab by antibody-dependent cellular cytotoxicity (ADCC), which is more advantageous as no proinflammatory fragments result from the complement activation. Furthermore, created apoptotic bodies are immediately engulfed by macrophages, also without any signs of inflammation induction [91].

Other two monoclonal antibodies have entered clinical trials: alemtuzumab, and daclizumab. Alemtuzumab *(Campath-1H)* is mAb-recognising CD52, the molecule expressed on T and B lymphocytes, natural killer (NK) cells, dendritic cells, monocytes, granulocytes, however, not on haematopoietic precursors. The biological role of CD52 seems to be in a participation of cell activation, at least in T lymphocytes. It was shown that CD52 cross-linking triggered their activation by induction of similar intracellular tyrosine phosphorylation events as employed by T cell receptor-mediated signalling. Furthermore, CD52 can serve as a costimulatory molecule involved in the induction of Treg cells [92, 93].

Treatment with alemtuzumab produces a very rapid and almost complete depletion of CD52-bearing cells in the circulation, mediated by ADCC [94, 95]. After depletion, repopulation of immune cells takes place differently. Monocytes return to normal values within three months; B cell counts return to baseline numbers also by three months and are then even increased to about 124% of pretreatment levels [91]. Increase of B cell counts is followed by enrichment in regulatory T cells. T cell counts recover much slower, as the depletion of CD4$^+$ cells lasts a median of 61 months and of CD8$^+$ cells for 30 months, respectively. The swift rise of B cells counts may explain a tendency of the alemtuzumab-treated patients to develop some autoimmune disorders, out of which the Graves' disease and autoimmune thrombocytopenia belong to the most severe [96, 97].

Alemtuzumab treatment of MS patients with relapsing-remitting forms of the disease has significantly reduced the risk of relapse and accumulation of disability, which suggested that it not only reduces disease activity due to the immune cell-depleting effect, but could perform other positive effects as well. Really, it was proved that it induced the production of neurotrophic factors in autoreactive T cells providing the CNS a neuroprotective effect. The group of Coles et al. showed that lymphocytes derived from alemtuzumab-treated MS patients produced enhanced amounts of brain-derived neurotrophic factor (BDNF) and ciliary neurotrophic factor (CNTF) upon antigen-specific stimulation with myelin basic protein (MBP) [98, 99].

Daclizumab is a humanised monoclonal antibody that binds to the alpha-chain of IL-2 receptor (CD25), thus effectively blocking the formation of its high-affinity form. Because the high-affinity IL-2 receptor signalling promotes the expansion of activated T cells *in vitro*, daclizumab was designed as a therapy that selectively inhibits T-cell activation and received approval as an add-on therapy to a standard immunosuppressive regimen for the prevention of acute allograft rejection in renal transplantation. Based on its mechanism of action, daclizumab represented an ideal therapy for T-cell-mediated autoimmune diseases too and was subsequently tested in the treatment of inflammatory uveitis and MS. In both of them, it significantly inhibited target organ inflammation. Subsequent studies of mechanisms of its action in MS resulted rather in a surprise; instead of inhibition of T-cell proliferation and production of cytokines, it was shown it had expanded and activated

immunoregulatory CD56^{bright} NK cells, which gained access to the brain parenchyma and killed autologous activated T cells [100, 101].

Ultimately, regarding the role of lymphocytes in the induction and therapy of MS, one should also mention a possibility to induce and expand patient's own Treg cells *in vitro* and subsequently reintroduce them to the patient [102]. Treg cells could block both the initiation of autoimmune responses and inhibit the function of established autoreactive effector cells. The system was successfully tested in the EAE model of MS. The studies have disclosed that transfer of MBP-reactive Treg cells prevented disease when given prior to immunisation and prevented relapses when administered after the onset of disease. The effect was seen only when relevant myelin antigen-specific Treg cells were transferred, but not with polyclonal Treg cells [46, 103]. This could represent a stumbling block to the possible use of Treg therapy in MS, where the relevant antigens are not well defined.

5. Laboratory Immunology and Clinical Practice

In recent years, we are witnesses of a substantial increase of our knowledge on particular immunopathological processes in MS, which has reflected in a better and more effective therapy that we shortly outlined in the previous paragraphs. However, there is still a question, which laboratory indicators should be taken into considerations when physicians are thinking over what type of treatment would fit best to a particular MS patient. The followup of particular population of T cells and their subsets in the peripheral blood surely informs a physician about response of the immune system to the therapy. For instance, level of expansion of CD56^{bright} NK cells and the decrease in ratios of T cells (as target cells) to CD56^{bright} NK cells (as effector cells) could represent a useful biomarker indicative of therapeutic response to daclizumab. However, our current knowledge of the great plasticity of T helper cells subsets and their ability of redifferentiation from one subset to another (e.g., T_H2 to T_H1, etc.) [104] will make us pay more attention to cytokines. If a cytokine profile is more tilted to a proinflammatory on the expense of an antiinflammatory, it will indicate that the pathological process is more intensive and our therapy is less efficient. For instance, the ratio between anti-inflammatory IL-10 and proinflammatory IL-12 correlates with the disease activity, for example, if patients respond to the IFN-β treatment, the IL-10 to IL-12 ratio increases [105].

The monitoring of levels of some adhesive and costimulatory molecules (VLA-4, LFA-1, VCAM-1, CTLA-4, and TIM-3) follows the same objective. For instance, if an MS patient responds to IFN-β treatment, the levels of his/her adhesive molecules in the peripheral blood are decreasing [105]. How to treat it, when to change the therapy, and whether a drug combination should be used still remain upon the physician's discretion. Furthermore, MS is not a uniform disease; on the contrary, it has its own subtle differences based on the predominance of one type of immunopathological process over the other, which prevails differently in every MS patient.

Obviously, the more we understand the underlying mechanisms and their interconnections, the more basic research will help physicians in their decisions.

List of Abbreviations

ADCC: Antibody-dependent cell-mediated cytolysis
APC: Antigen-presenting cells
BBB: Blood-brain barrier
BLIMP-1: B-lymphocyte-induced maturation protein
CMV: Cytomegalovirus
CTLA: Cytotoxic T lymphocyte antigen
DC: Dendritic cell
EAE: Experimental autoimmune encephalomyelitis
EBV: Epstein-Barr virus
FOXO: Forkhead box O
FOXP3: Forkhead box P
GITR: Glucocorticoid-induced tumour necrosis factor receptor family-related gene
GM-CSF: Granulocyte-macrophage colony-stimulating factor
GvHD: Graft versus host reaction disease
HBV: Hepatitis B virus
HLA: The major histocompatibility complex in man
HSV: Herpes simplex virus
IFN-β/IFN-γ: Interferon beta/gamma
IL: Interleukin
IL-1Ra: Interleukin 1 receptor antagonist
IRF4: Interferon regulatory factor 4
mAb: Monoclonal antibodies
MBP: Myelin basic protein
MOG: Myelin oligodendrocyte antigen
NK: Natural killer cells
PD: Programmed death
PLP: Proteolipid protein
STAT5: Signal transducer and activator of transcription 5
TCR: T-cell receptor
TIGIT: T-cell Ig and ITIM domain
TIM: T-cell immunoglobulin mucin protein.

Acknowledgment

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Clinical Study

Elevated Osteopontin Levels in Mild Cognitive Impairment and Alzheimer's Disease

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Inflammatory mediators are closely associated with the pathogenesis of neurodegenerative changes in Alzheimer's disease (AD) and mild cognitive impairment (MCI). Osteopontin (OPN) is a proinflammatory cytokine that has been shown to play an important role in various neuroinflammatory diseases. However, the function of OPN in AD and MCI progression is not well defined. Cerebrospinal fluid (CSF) and plasma samples were obtained from 35 AD patients, 31 MCI patients, and 20 other noninflammatory neurologic diseases (OND). Concentrations of OPN in the CSF and plasma were determined by enzyme-linked immunosorbent assay. During a 3-year clinical followup, 13 MCI patients converted to AD (MCI converters), and 18 were clinically stable (MCI nonconverters). CSF OPN concentrations were significantly increased in AD and MCI converters compared to OND, and increased levels of OPN in AD were associated with MMSE score; OPN protein levels both in the CSF and plasma of newly diagnosed AD patients were higher than that of chronical patients. In MCI converters individuals tested longitudinally, both plasma and CSF OPN concentrations were significantly elevated when they received a diagnosis of AD during followup. Further wide-scale studies are necessary to confirm these results and to shed light on the etiopathogenic role of osteopontin in AD.

1. Introduction

Alzheimer's disease (AD) is a prominent neurodegenerative disorder characterized by chronically progressive global cognitive decline [1]. Mild cognitive impairment (MCI) has been recognized as intermediate between normal elderly cognition and dementia. A proportion of individuals with MCI may progress to AD and have typical biological changes of this kind of dementia [2]. To date, the underlying mechanisms involved in the degeneration of cerebral neurons and synapses in AD remain an enigma, but the theory about the involvement of inflammatory processes and immune dysregulation in their pathogenesis has been widely demonstrated. Neuropathological and neuroradiological studies have demonstrated that inflammatory changes in AD brains are a relatively early pathogenic event that precedes the process of neuropil destruction [3, 4]. In addition, studies have demonstrated that the brains and CFS of AD and MCI contained various proinflammatory substances, such as cytokines, acute phase proteins, and complement proteins [5–7], which have a fundamental role in inducing cognitive decline, memory loss, and dementia. It has been proposed that the autoimmune mechanism may be a trigger for AD, which was confirmed by the presence of autoantibodies and the apparently good outcome after immunotherapy as seen both in the animal model and in a few patients tested. Circulating autoantibodies against $A\beta$ are several times higher in individuals with AD, and antibody titers correlate with cognitive dysfunction [8]. Active immunization and the use of monoclonal antibodies for passive vaccination have provided encouraging data in transgenic mouse models of AD [9].

Osteopontin (OPN), also called "early T cell-activation gene 1" [10], is a negatively charged acidic hydrophilic protein that is produced by various cell types and participates in diverse physiological and pathological processes,

TABLE 1: Clinical details and laboratory findings of HC, OND, MCI, and AD patients.

	HC	OND	MCInc	MCIc	ADn	ADc
Individuals, no.	20	20	18	13	17	18
Gender, no. of female/male	10/10	11/9	8/10	9/4	11/6	8/10
Mean age (mean ± SD)	72 (57–81)	71 (58–82)	73 (59–83)	72 (60–82)	73 (61–81)	74 (59–83)
MMSE score (mean ± SD)	29 ± 1.5	28 ± 1.7	27.1 ± 1.4	27.9 ± 1.6	22.9 ± 2.7	18.9 ± 4.3
OPN plasma levels (ng/mL) (mean ± SD)	51.4 ± 9.8	53.3 ± 10.3	52.3 ± 11.7	53.7 ± 11.1	74.4±13.7	54.8 ± 10.1
OPN CSF levels (mean ± SD)	—	134.5 ± 19.3	128.6 ± 17.7	173.2 ± 20.6	226.5 ± 21.2	165.6 ± 20.4
Aβ CSF levels (pg/mL) (mean ± SD)	—	326.7 ± 45.8	361.2 ± 48.1	181.7 ± 32.6	167.8 ± 26.9	196.3 ± 29.6
Tau CSF levels (pg/mL) (mean ± SD)	—	112 ± 21.6	141 ± 25.8	243.7 ± 31.6	275.4 ± 34.9	289.6 ± 38.9

HC: healthy control; OND: non-inflammatory neurologic diseases; MCInc: MCI non-converters; MCIc: MCI converters; Adn: newly diagnosed AD; ADc: chronical AD; OPN: osteopontin; SD: standard deviation.

including bone mineralization, oxidative stress, remyelination, wound healing, inflammation, and immunity [11–13]. Studies have demonstrated marked upregulation of OPN in various inflammatory and autoimmune diseases. The expression of OPN was elevated in the brains of rats with experimental autoimmune encephalomyelitis (EAE) but not in brains of rats protected from EAE, and severity of EAE was significantly reduced in OPN deficient mice [14]. In concordance with those findings in animal models, OPN transcripts were frequently detected and were exclusive to the multiple sclerosis mRNA population, but not found in control brain mRNA [15]. In addition, the expression levels of OPN in plasma and tissues are also elevated in other several inflammatory or autoimmune disorders, such as rheumatoid arthritis, inflammatory bowel disease, systemic lupus erythematosus, and lymphoproliferation disease [16–19]. It has been well studied that interactions between OPN and its receptors (including $\alpha v \beta 3$, $\alpha 5 \beta 1$ and CD44) mediated survival, migration, and adhesion in many types of cells [20, 21]. As a proinflammatory mediator, OPN plays a role in the progression of chronic inflammatory and autoimmune diseases through various mechanisms, including involving in generation of Th1 and Th17 cells that are pathogenic T cells for various inflammatory diseases [22–24], inhibiting apoptosis of autoreactive immune cells and recruitment of leukocytes to sites of inflammation [21, 25].

Recently, the important role of OPN in AD has been investigated both in humans and animals model [26]. In AD brains, there was a significant 41% increase in the expression of OPN in pyramidal neurons compared with age-matched control brain, and there was a significant positive correlation between OPN staining intensity and amyloid-beta load [27]. By means of proteomic analysis of CSF samples, Simonsen and colleagues identified a phosphorylated C-terminal fragment of OPN that was increased in patients with MCI progressing to AD as compared to patients who remain stable over time and healthy controls [28]. Comi et al. demonstrated that OPN levels are increased in the CSF of AD subjects as compared to controls and its levels are

more markedly raised in the early stages of the disease and correlate with cognitive decline [29]. In addition, upregulated OPN expression has also been demonstrated in AβPP/PS1 KI mice, an animal model of AD with severe pathological alterations [30]. Collectively, these findings strongly suggest the involvement of OPN in the development of AD.

To further clarify the role of OPN in the progression of cognitive decline, we longitudinally assessed the OPN expression changes in the plasma and CSF in the same individuals. On the other hand, we transversely analyzed OPN levels in patients with MCI, newly diagnosed AD, and chronical AD, comparing the results with those obtained in the groups of healthy control and other noninflammatory neurologic diseases (OND).

2. Materials and Methods

2.1. Patients. Thirty-five patients affected by AD and Thirty-one patients with a diagnosis of MCI were selected for the study. Table 1 lists the demographic data of the subjects, including gender, age, and the Mini-Mental State Examination Score (MMSE) [31], which is a general measure of cognitive performance.

The clinical diagnosis of AD was performed according to NINCDSADRDA work group criteria and DSM IV-R [32, 33]. The mean age of AD patients (16 males and 19 females) was 78.2 years (age range 58–80 years). All patients underwent complete medical and neurological evaluation, laboratory analysis, CT scan, or MRI to exclude reversible causes of dementia. Standard laboratory tests performed at the time of diagnosis included complete blood count, serum electrolytes, serum glucose, blood urea nitrogen, B12, folate, thyroid function tests, and serology for syphilis. Neuropsychological evaluation and psychometric assessment was performed with a Neuropsychological Battery including MMSE, Digit Span Forward and Backward, Logical Memory and Paired Associated Words Tests, Token Test, Supra Span Corsi Block Tapping Test, Verbal Fluency Tasks, Raven Colored Matrices,

the Rey Complex Figure, Clinical Dementia Rating Scale (CDR) [34], and the Hachinski Ischemic Scale. Thirty-two patients were late and three early AD were onsets; all cases were sporadic. MMSE and CDR scales were used to assess the severity of dementia. AD patients were divided into two subgroups according to disease duration: 17 newly diagnosed AD patients (ADn, disease duration $\leq$ 2 years) and 18 chronical AD patients (ADc, disease duration > 2 years). The mean MMSE for AD group was 21.2 + 1.8 (scores 6–27).

The diagnosis of MCI was based on the following unanimously adopted criteria: (1) reported cognitive decline; (2) impaired cognitive function; (3) essentially normal functional activities; and (4) exclusion of dementia [35, 36]. The mean age of MCI patients (17 males and 14 females) was 74.2 years (age range 57–82). All of these patients received neurological examination, laboratory test, and brain MRI to exclude intracranial mass, infarcts, moderate to severe nonspecific white matter disease, and reversible causes of cognitive impairment. The same neuropsychological battery discussed in the AD section was used for MCI patients. All patients had a follow-up visit every 6 months, and the monitoring period was 3 years. Based upon subsequent diagnosis status at follow-up evaluations, MCI participants can be divided into two subgroups: 13 MCI patients who have converted to AD (MCI converters, MCIc) and 18 MCI patients who have not converted to AD (MCI nonconverters, MCInc). The mean MMSE for MCI group was 27.6 ± 1.6 (scores 25–29).

Twenty patients with OND (11 females and 9 males, age 59–79 years, mean age 76.4 ± 13.1 years) were also enrolled in the study. These patients with the following conditions: 2 strokes, 5 transient ischemic attacks, 4 chronic intractable headache, 3 status epilepticus, 3 normal pressure hydrocephalus, and 3 peripheral neuropathies. Plasma samples were also obtained from 24 healthy elderly subjects (HC), age and sex matched with the patient (11 males and 10 females, age 57–81 years, mean age 74.7 ± 14.5 years). These individuals were either unrelated healthy spouses of AD and MCI patients or healthy volunteers, and they had no family history of dementia or evidence of acute or chronic diseases at the time of enrollment. The cognitive status of OND and HC was assessed by administration of MMSE (score for inclusion as normal control subjects $\geq$ 28). All formal neurocognitive test scores for these participants were within 1.5 standard deviations of normative data in published studies or manuals.

Patients with an inflammatory or infectious disease, with a history of immunological or malignant disease, medication of immunologically relevant drugs, abnormal white blood cell count, and abnormal CSF findings, were not included into the study. All study procedures were approved by the Harbin Medical University, China, Institutional Review Board, and all participants or their representatives gave informed consent. All of blood and CSF samples were obtained at the initial visit. In those MCI converters, the second blood and CSF samples were available when they received a diagnosis of AD during followup.

2.2. CSF and Plasma Samples. After lumbar puncture, CSF samples (20–30 mL) were obtained and collected in polypropylene tubes. The samples were centrifuged at 2,000 g at 4°C for 10 minutes to eliminate cells and other insoluble material and were then immediately frozen and stored at −80°C pending biochemical analyses, without being thawed or refrozen. Cell count was performed on the CSF samples and no sample contained more than 500 erythrocytes/μL. Ethylenediamine tetraacetic acid (EDTA) or citrate plasma was obtained by venous puncture. Plasma was isolated by centrifugation and stored at −80°C until use.

2.3. Determination of OPN in Plasma and CSF. The OPN protein content in serum and CSF was measured using a commercial ELISA according to the manufacturer's instructions (Assay Designs, Inc., Ann Arbor, MI, USA). Briefly, serum and CSF samples were diluted, respectively, 1 : 20 and 1 : 50 into Assay Buffer provided by the manufacturer and were incubated at 37°C for 1 h in microtiter plates precoated with a polyclonal N-terminal capture anti-OPN antibody (Assay Designs). Then, the plates were washed and wells were incubated at 4°C for 30 min with a horseradish peroxidase labeled OPN-specific monoclonal antibody (Assay Designs). After washing, the wells were incubated with tetramethylbenzidine-H_2O_2 solution for 30 min. The color reaction was stopped by adding a solution containing 1 N sulfuric acid. Optical densities were measured at 450 nm with reference wavelength set at 590 nm. The OPN concentrations were calculated using a standard curve of recombinant human OPN provided by the manufacturer. The lower detection limit of the kit was 3.33 ng/mL. Baseline and follow-up CSF and plasma samples from a patient were measured on the same plate. Assays were repeated when the difference between the two probes of one sample was more than 10%.

2.4. Statistical Analysis. Normally distributed data sets were analysed by Student's t-test, paired t-test, analysis of variance (ANOVA), and linear regression and correlation analysis (using "Primer for Biostatistics"). $P < 0.05$ was considered significant.

3. Results

3.1. OPN Levels in Plasma. The demographic characteristics of the patient material are given in Table 1. There was no difference in sex distribution in MCI, AD, and OND patients and the healthy control subjects.

Plasma OPN concentrations in healthy controls (51.4 ± 9.8 ng/mL) and OND (53.3 ± 10.3 ng/mL) did not differ significantly and did not correlate with age and sex. Plasma OPN concentrations in MCInc (52.3 ± 11.7 ng/mL), MCIc (53.7 ± 11.1 ng/mL), ADn (74.4 ± 13.7 ng/mL), ADc (54.8 ± 10.1 ng/mL), OND patients, and healthy controls differed significantly (Figure 1(a); $P < 0.001$). ADn patients had higher plasma concentrations of OPN than the healthy controls ($P < 0.005$), whereas plasma concentrations of OPN in the MCInc, MCIc, and ADc patients did not differ significantly from plasma OPN concentrations in OND or healthy controls (Figure 1(a)).

(a)

(b)
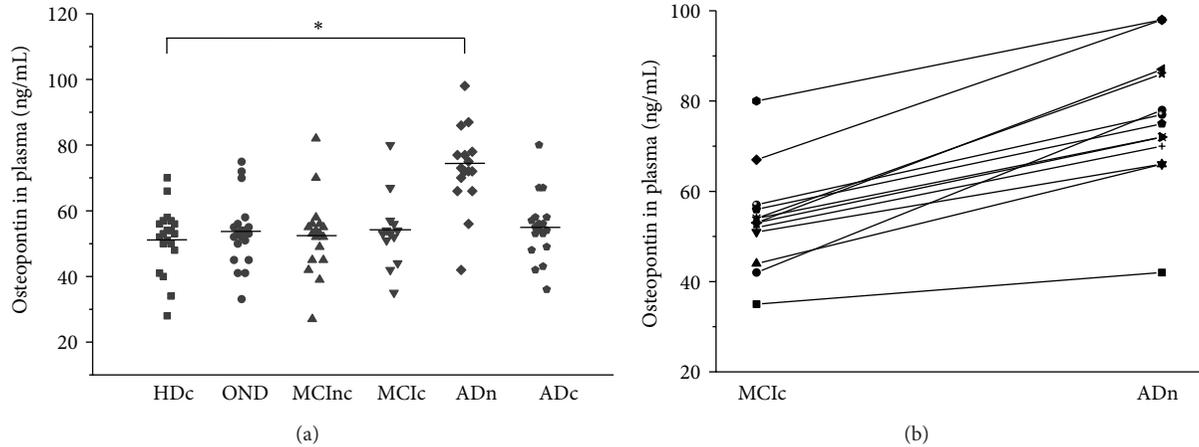
FIGURE 1: Plasma osteopontin levels in newly diagnosed Alzheimer's disease (AD) are elevated. The horizontal lines represent the mean values of each group, and asterisks show statistical significance ($^*P < 0.01$). (a) Osteopontin concentrations in plasma of 20 healthy control (HC), 20 aged-matched other noninflammatory neurologic diseases (OND), 18 clinically stable mild cognitive impairment patients (MCInc), 13 mild cognitive impairment converters (MCIc), 17 newly diagnosed AD (ADn), and 18 chronical AD patients (ADc) were determined using an enzyme-linked immunosorbent assay (ELISA). (b) The plasma osteopontin concentrations within the same individual ($n = 13$) at different disease statuses.


(a)

(b)
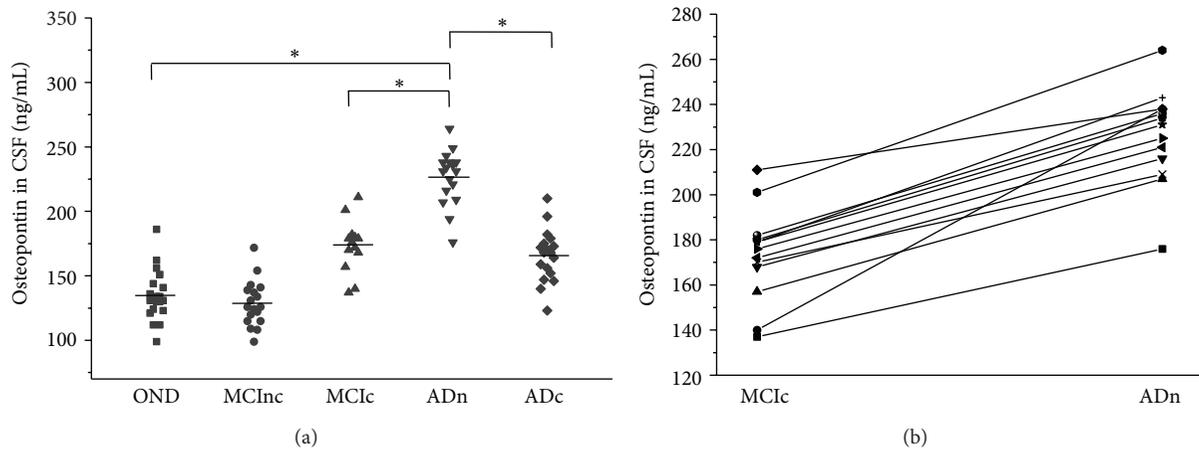
FIGURE 2: Osteopontin protein levels in the cerebrospinal fluid (CSF) of each group patients. The horizontal lines represent the mean values of each group, and asterisks show statistical significance ($^*P < 0.01$). (a) Osteopontin concentrations in CSF of 20 other noninflammatory neurologic diseases (OND), 18 clinically stable mild cognitive impairment patients (MCInc), 13 mild cognitive impairment converters (MCIc), 17 newly diagnosed AD (ADn), and 18 chronical AD patients (ADc) were determined using an enzyme-linked immunosorbent assay (ELISA). (b) The CSF osteopontin concentrations within the same individual ($n = 13$) at different disease statuses.

We next questioned whether the plasma OPN concentrations would vary within the same individual in relation to disease status. In 13 MCIc individuals tested longitudinally, the plasma OPN concentrations were significantly elevated when they received a diagnosis of AD during followup (Figure 1(b)).

3.2. OPN Levels in CSF.
The CSF represents the fluid compartment that is closest to reflect the inflammatory situation in the degenerative processes of the nervous system, so we then sought to compare the concentrations of OPN in CSF from patients with MCI, AD, and OND.

CSF OPN concentrations differed significantly in MCInc (128.6 ± 17.7 ng/mL), MCIc (173.2 ± 20.6 ng/mL), ADn (226.5 ± 21.2 ng/mL), ADc (165.6 ± 20.4 ng/mL), and OND patients (134.5 ± 19.3 ng/mL) (Figure 2(a)). Patients with MCIc, ADn, and ADc ($P < 0.001$) had significantly higher OPN concentrations in the CSF than the neurological controls. The concentrations of OPN in CSF from patients with ADn was significantly higher than that from patients with ADc ($P < 0.001$). CSF concentrations of OPN did not differ in MCInc and OND patients.

We also determined whether the CSF OPN concentrations would vary within the same individual in relation to disease status. In 13 MCIc individuals tested longitudinally, the CSF OPN concentrations were significantly elevated when they received a diagnosis of AD during followup (Figure 2(b)).
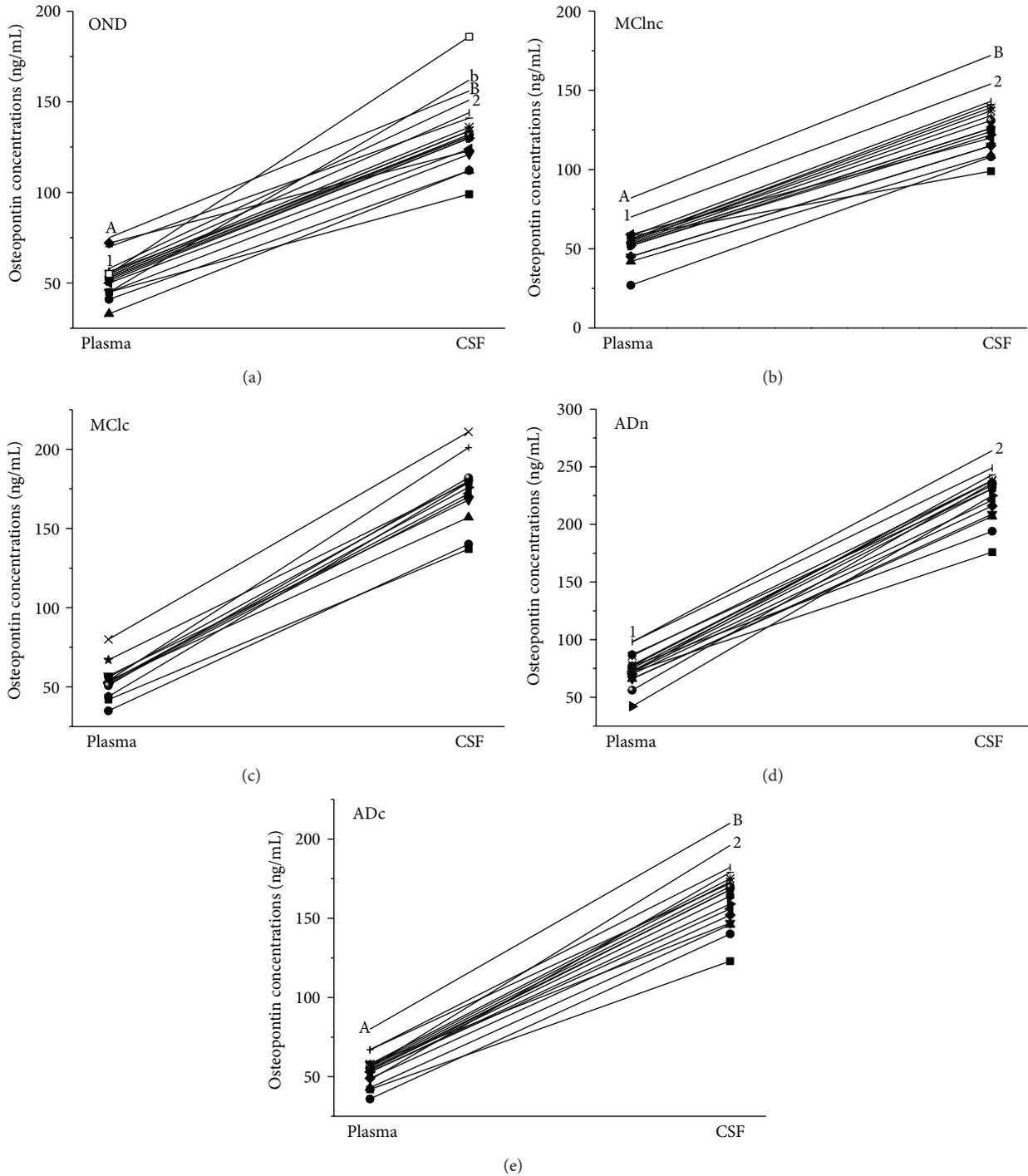
FIGURE 3: The concentrations of osteopontin in the cerebrospinal fluid (CSF) were significantly higher compared to peripheral blood in patients with other noninflammatory neurologic diseases (OND, $n = 20$), clinically stable mild cognitive impairment patients (MCInc, $n = 18$), mild cognitive impairment converters (MCIc, $n = 13$), newly diagnosed AD (ADn, $n = 17$), and chronical AD patients (ADc, $n = 18$).

When comparing paired CSF and blood samples from patients with MCI, AD, and OND, the concentrations of OPN in the CSF were significantly higher than plasma concentrations in all patients (Figure 3).

3.3. *Correlation between CSF OPN Concentrations and Clinical Parameters in Patients with AD.* First, we determined whether there was a correlation between the levels of OPN in the CSF and the degree of cognitive decline in AD

(a)

(b)
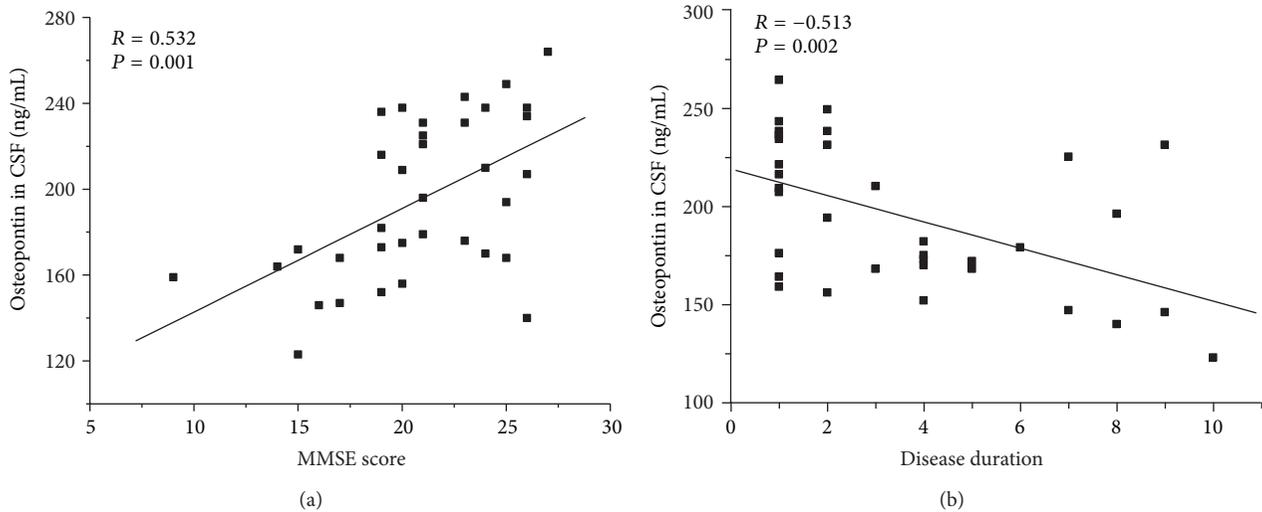
FIGURE 4: Correlation coefficients were computed to evaluate the relationship between cerebrospinal fluid (CSF) osteopontin levels and the degree of cognitive decline or disease duration in AD patients. (a) A positive correlation between CSF osteopontin levels and the Mini-Mental State Examination Score (MMSE) score in AD patients. (b) The CSF OPN concentration was correlated inversely with the disease duration.

patients. A strongly positive correlation between the CSF OPN concentrations and the MMSE score ($r = 0.53$, $P < 0.001$) was observed (Figure 4(a)). Secondly, we explored whether there was a correlation between the levels of OPN in the CSF and disease duration of AD patients. Our result showed that the CSF OPN concentration was correlated inversely with the disease duration ($r = -0.51$, $P < 0.001$) (Figure 4(b)).

No other clinical parameters, such as gender, age, Aβ42, tau, and P tau levels, had any significant correlation with the levels of OPN in the CSF of AD patients.

Although a trend was noted toward the positive correlation between the plasma OPN concentrations and the MMSE score in the ADn patients, it was not statistically significant.

By contrast, no clear correlation was found between the CSF OPN concentrations of MCIc and both the degree of cognitive decline and disease duration.

4. Discussion

In this study, the crosswise comparison demonstrated that CSF OPN concentrations were significantly increased in AD and MCI converters compared to OND, and OPN protein levels both in the CSF and plasma of newly diagnosed AD patients were higher than that of chronical patients. Furthermore, in MCI converters individuals tested longitudinally, both plasma and CSF OPN concentrations were significantly elevated when they received a diagnosis of AD during followup. Finally, OPN CSF levels displayed direct correlation with the MMSE score and inverse correlation with disease duration.

Our data have shown that the plasma concentrations of OPN were significantly increased in the group of newly diagnosed AD than the other groups, and there was a trend toward the positive correlation between the plasma OPN concentrations and the MMSE score, although it was

not statistically significant. One previous study on serum OPN concentrations in AD patients gave a different result from ours. Comi et al. found that there were no significant differences in serum OPN concentrations comparing AD to controls [29]. The results of the present study suggest that the differences between the results of the previous study may, at least partly, be explained by that we stratified patients according to their disease duration. Studies demonstrated that high levels of plasma OPN were well correlated with the activities of various disease conditions: plasma OPN concentrations were significantly higher in systemic lupus erythematosus patients and increase in OPN concentration correlated positively and significantly with SLEDAI score in all patients [18]; serum OPN concentrations of patients with idiopathic retroperitoneal fibrosis were elevated compared to healthy controls and correlated with the transverse diameter of the periaortic cuff as determined by imaging studies [37]. In addition, elevated plasma OPN levels have been also shown to play an important role in inflammatory and degenerative processes of the central nervous system: OPN plasma levels were elevated in secondary progressive MS compared to relapsing-remitting MS patients in remission and healthy controls, supporting a role for OPN in the chronic disease activity [38]; OPN serum levels were elevated in Parkinson's disease and higher serum levels were associated with more severe motor symptoms [39].

Prospective epidemiological studies show that elevated plasma levels of acute phase reactants can be considered as a risk factor for AD. Therefore, we speculate that elevated OPN plasma levels in the initial stages of AD may contribute to the progression of cognitive decline, although the exact role of OPN and its underlying mechanism as a key proinflammatory cytokine in AD is not understood. Th17 cells have been shown to be a pathogenic effector cell for development of various inflammatory and autoimmune diseases. Shinohara and colleagues found that OPN plays a critical positive role in

the differentiation of Th17 cells by repressing IL-27 secretion in mouse dendritic cells [40]. A recent report showed that Th17 T cells were increased in AD patients [41], which favors the speculation that elevated OPN plasma levels in AD may be associated with the differentiation of Th-17 cells.

Accumulating evidence suggests that inflammation mainly occurs in pathologically vulnerable regions of the AD brain (such as the entorhinal, temporoparietal, and cingulate cortex), with increased expression of acute phase proteins and proinflammatory mediators [42]. Therefore, we further examined the concentrations of OPN in the CSF, which directly contact with brain and can accurately reflect the ongoing inflammatory process in the central nervous system. Our results showed that the CSF concentrations of OPN were significantly increased in patients with MCI progressing to AD than that in stable MCI. Prediction of conversion from MCI to AD is of major interest in AD research, which would allow for the appropriate application of disease-modifying treatments at a point where clinical manifestations are limited. Presently, there are few clinical or imaging markers for the early identification of MCI which progresses to AD and MCI which does not progress. Recently, Simonsen and colleagues found that a phosphorylated C-terminal fragment of OPN was increased in the CSF of patients with MCI progressing to AD as compared to patients who remain stable over time and healthy controls and proposed OPN as a biomarker to predict the progression of MCI to overt AD [28]. The findings of these two studies showed that not only the intact forms of OPN but also the cleaved form of OPN was increased in the CSF of patients with MCI progressing to AD. More importantly, our results showed that the CSF OPN concentrations of MCIc were significantly elevated when they received a diagnosis of AD during followup. Our findings are in agreement with another study, which showed that AD patients displayed about a two-fold increased OPN levels in the CSF compared to age-matched controls and it was particularly striking in the early stages of the disease [29]. To identify protein changes during the presymptomatic phase of AD, Ringman and colleagues performed proteomic analyses of CSF from persons with or at risk for inheriting familial AD using high-resolution liquid chromatography-mass spectrometry [43]. Their results showed that OPN was elevated in the CSF of familial AD mutation carriers compared to related noncarriers, which suggest changes of OPN in the CSF occurring a decade before clinical dementia. In addition, increased serum and CSF OPN levels were also detected in the Lewy dementia, and the genotypic variation of SNP-66 was associated with the occurrence of the Lewy body dementia [44]. These findings suggest that OPN is associated with the occurrence of cognitive decline.

Studies showed that OPN seemed to act as a double-edged and might exert two opposite functions in the progression of neurodegenerative diseases. On one hand, OPN functions as a neuroprotectant by upregulating myelination and remyelination. On the other hand, OPN had a disease-accelerating role by triggering neuronal toxicity and death. In the current study, though we could not directly conclude that the increased levels of OPN within the CSF stimulate the degeneration of cerebral neurons and synapses in

AD, we conjecture that OPN may favor AD development because MCIc patients had high levels of CSF OPN and the concentrations of OPN in the CSF and plasma were further increased when they received a diagnosis of AD. Our speculation is supported by the study of Wung and collaborators. Their results demonstrated that OPN expression was increased in the pyramidal neurons of the CA1 region of the hippocampus of AD patients and OPN staining intensity positively correlated with both amyloid-beta load and age [27]. Increased OPN expression may exacerbate the abnormal immune response presented in the AD brain by enhancing the survival of activated T cells, which were detected in the brain tissues of AD patients [45].

In conclusion, the bell-shaped curve of CSF OPN expression in disease progression of cognitive decline has extended the evidence for a role of OPN in AD pathogenesis. It will be important to study larger cohorts of individuals with longer durations of followup, and from different centers, to further evaluate whether higher baseline level of CSF OPN was associated with a more marked decline of MMSE over followup.

Conflict of Interests

The authors have no conflict of interests.

Authors' Contribution

Y. Sun, X. S. Yin, H. Guo, R. K. Han and R. D. He contributed equally to the work.

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Clinical Study

Eosinophil-Derived Neurotoxin Is Elevated in Patients with Amyotrophic Lateral Sclerosis

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Background and Objectives. Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease characterized by loss of motor neurons in the brainstem, motor cortex, and spinal cord. Oxidative stress and neuroinflammation have been implicated in the pathophysiology of ALS. Members of the family of damage-associated molecular patterns, including reactive oxygen species, high-mobility group box 1, and eosinophil-derived neurotoxin (EDN), may participate in pathological conditions. In this study, we aim to discover new biomarker for detecting ALS. *Materials and Methods*. We examined 44 patients with ALS, 41 patients with Alzheimer's disease, 41 patients with Parkinson's disease, and 44 healthy controls. The concentration of serum EDN was measured using an enzyme-linked immunosorbent assay. *Results*. EDN levels were significantly increased 2.17-fold in the serum of patients with ALS as compared with healthy controls ($P < 0.05$). No correlation between the levels of serum EDN and various clinical parameters of ALS was found. Moreover, the levels of serum EDN in patients with Parkinson's disease and Alzheimer's disease and healthy controls were similar. *Conclusion*. A higher level of serum EDN was found specifically in patients with ALS, indicating that EDN may participate in the pathophysiology of ALS.

1. Introduction

Amyotrophic lateral sclerosis (ALS) is the most common and devastating adult-onset neurodegenerative disease [1]. The underlying pathology involves the selective loss of motor neurons in the spinal cord, brainstem, and cerebral cortex [2]. Weakness and muscle atrophy are the typical earliest symptoms of ALS and are followed by rapid progression leading to total paralysis and respiratory failure within 2 to 5 years after diagnosis. Generally, ALS is considered to be a neuromuscular disorder, but more studies are beginning to recognize ALS as a multisystem neurological disease [3–5]. The annual incidence of ALS is 1-2 per 100,000 persons [6]. There are two types of ALS. One is familial ALS, which accounts for

only 10% of all ALS cases [7]. A known hereditary factor, mutant Cu/Zn superoxide dismutase 1 (SOD1), is associated with ~20% of cases of familial ALS. The second type of ALS is sporadic ALS, the cause(s) of which is less well understood.

Currently, the etiology of ALS is unclear. However, several lines of evidence suggest that neuroinflammation [8], glutamate excitotoxicity [9], altered cytoskeletal proteins [10], impaired axoplasmic transport [11], and oxidative stress [12] are involved in disease development. Oxidative stress is believed to play an important role in ALS. Some SOD1 mutations such as SOD1-G93A, SOD1-G85R, and SOD1-G37R lead to a loss of dismutase activity in transgenic mice, resulting in the accumulation of high concentrations of reactive oxygen species (ROS) in motor neurons [13]. Subsequently, the free

radicals generated by superoxide may damage neurons. Moreover, as motor neurons encounter excessive oxidative stress, high levels of cytoprotective heat shock proteins (HSPs) may be induced [14]. Generally, overexpression of HSPs is associated with cellular stress responses including heat shock [15], heavy metal stress [16], and disease [17]. A recent ALS study has indicated that the serum levels of HSPs are elevated in patients with ALS and in ALS mouse models [14]. Expression of protective proteins suggests that ALS may result from pathophysiological stress such as neuroinflammation. Neuroinflammation has been widely discussed as a mechanism of ALS [18]. During chronic neuroinflammation, activated microglia play direct and indirect destructive roles in inducing the expression and release of cytokines such as tumor necrosis factor-α, which stimulates local inflammatory responses and neuronal degeneration [19, 20]. Furthermore, microglia also secrete proinflammatory factors such as chemokines and ROS, which contribute to neuroinflammatory processes [21, 22]. Microglia may attack neurons, inducing progressive cell loss in specific neuronal populations in neurodegenerative disorders [23–25]. Therefore, factors that induce inflammatory responses could serve as potential biomarkers. Serum levels of the inflammatory alarm protein, high-motility group box 1 (HMGB1), have been reported to be overexpressed in the spinal cord and brain in an ALS mice model and in biopsies from patients with ALS [26, 27]. Because these prevalent damage factors and danger signals are involved in ALS, we hypothesized that two signals belonging to the danger signal family, eosinophil-derived neurotoxin (EDN) and eosinophil cationic protein (ECP), may be correlated with ALS.

EDN, also known as RNase2, is a member of the ribonuclease A superfamily [28]. EDN is a single-chain polypeptide with an observed molecular mass of 18.6 kDa. EDN is expressed mainly in eosinophils but is also detected in mononuclear cells and possibly neutrophils [29]. EDN possesses full ribonucleolytic activity and is involved in defending the upper bronchial tract from infection by respiratory syncytial virus [30, 31]. In addition, EDN is likely to be a host molecule that may induce proinflammatory cytokine production in monocyte/macrophages and the maturation of dendritic cells through Toll-like receptor 2 (TLR2) [32]. Furthermore, EDN causes serious damage to myelinated neurons in the rabbit brain, an event known as the Gordon phenomenon [33–35]. Damage to Purkinje cells and devastating spongiform degeneration in the white matter of the brainstem, cerebellum, and spinal cord are also caused by EDN [36]. Therefore, it is rational to suggest that EDN plays a critical role in neuronal damage and is involved in the loss of neurons, resulting in neurodegenerative disorders.

ECP is a paralog of EDN in humans, and they share 70% similarity at the protein level. They are both secreted by activated eosinophils during pathogenic stimulation and inflammatory processes [37]. In patients with asthma, the serum level of ECP is elevated and serves as a clinical biomarker for monitoring asthma severity [38]. In pathophysiological conditions, accumulation of ECP induces chronic inflammation and enhances the severity of inflammation such as that which occurs during the inflammation of the intestinal mucosa in

Crohn's disease [39]. The tissue damage attributed to ECP depends on its interaction with the organism surface, which occurs during its translocation into the cell. The hypothesized mechanism of ECP-triggered cell damage is that ECP destabilizes the cell membrane via the processes of pore formation, permeability changes, and membrane leakage [40].

Because EDN and ECP participate in the induction of inflammatory diseases and because both serve as disease biomarkers, we examined the serum levels of EDN and ECP in patients with ALS.

2. Materials and Methods

2.1. Participants. The demographic information for the normal control individuals and the patients with ALS is given in Table 1. Forty-four patients with ALS (25 male, 19 female), 39 patients with Alzheimer's disease (AD; 16 male, 23 female), 40 patients with Parkinson's disease (PD; 20 male, 20 female), and 44 age-matched, unrelated healthy controls (24 male, 20 female) were recruited by the Taipei City Hospital Zhongxiao Branch, Taipei, Taiwan. Informed consent was obtained before blood sampling, and all procedures were approved by the Institutional Review Board of Taipei City Hospital. All patients and controls were Taiwanese. The clinical severity of patients with ALS was evaluated using the Amyotrophic Lateral Sclerosis Functional Rating Scale-Revised (ALSFRS-R) ranged from 0 to 47. The mean and SD of ALSFRS-R in this study were 17.8 and 13.28, respectively. The disease duration of patients with ALS ranged from 2 to 84 months (mean = 13.6 months, SD = 14.65), and the onset types were classified as brainstem (B), hand (H), and foot (F).

2.2. Enzyme-Linked Immunosorbent Assay (ELISA). Venous blood (10 mL) was collected from patients with ALS, AD, and PD as well as controls; all participants were free of acute infection and acute stress at the time of collection. Serum collected using yellow-stopper clot-accelerating tubes was harvested by centrifugation at $3000 \times g$ for 20 min, divided into aliquots, and frozen at $-30°C$ until use. The serum levels of EDN and ECP were measured using commercially available ELISA kits for human EDN and ECP (both kits from MBL, Naka-Ku, Nagoya, Japan).

2.3. Statistical Analysis. The serum concentrations of EDN and ECP were compared among patients with ALS, AD, and PD and controls using an ANOVA. The one-way ANOVA test was used to determine the difference among all of the test groups. When the P value of the ANOVA reaches the significance level, Bonferroni multiple comparison test was used to determine the significant difference among the groups of control, AD, PD, and ALS. The Spearman rank correlation was used to correlate the levels of EDN or ECP versus the subgroups of age, ALSFRS-R, disease duration, or onset types.

3. Results

3.1. EDN Is Elevated in Sera of Patients with ALS. The concentration of serum EDN in patients with ALS, AD, and PD

TABLE 1: Study population demographics and clinical characteristics.

| | N | Age (years)[a] | | | Gender | ALSFRS-R[b] | Disease duration[c] | Type of onset |
		Mean (SD)	Range	Median	(M/F)	Mean (SD)	Mean (SD)	(B/H/F)[d]
Control	44	60 (10.10)	30–87	65	23/17	—	—	—
AD	39	80 (7.71)	54–90	81	16/23	—	—	—
PD	40	76 (9.17)	53–90	77	20/20	—	—	—
ALS	44	58 (9.88)	34–79	57	25/19	17.8 (13.29)	13.6 (14.65)	13/12/19

[a] Age was at the time of blood collection.
[b] The ALSFRS-R is a scale from 0 to 48 which assesses disability in patients with motor neuron diseases. 0 means serious and 48 means normal.
[c] The disease duration indicates months since the onset of symptoms.
[d] B: brainstem, H: hand, F: foot.

and in healthy controls was measured using a commercial ELISA kit. The average EDN levels in patients with ALS and controls were 45.7 ng/mL (SD = 29.3 ng/mL; range, 8.9–140.9 ng/mL) and 21.0 ng/mL (SD = 14.9 ng/mL; range, 8.6–79.4 ng/mL), respectively (Table 2). The level of EDN was significantly increased by 2.17-fold in the sera of patients with ALS as compared with the control group ($P < 0.005$; Figure 1(a)). The serum EDN level in patients with ALS was significantly increased 1.61- and 1.84-fold as compared with patients with AD (28.3 ng/mL; SD = 36.6 ng/mL; range, 1.9–158.0 ng/mL) and PD (24.8 ng/mL; SD = 20.7 ng/mL; range, 3.1–95.5 ng/mL), respectively (Figure 1(a)). These data indicate that serum EDN is specifically elevated in patients with ALS and may serve as an indicator for ALS.

Next, the ALSFRS-R, disease duration, age, and disease onset among patients with ALS were analyzed and statistically correlated with EDN levels. The clinical indicators were not correlated with EDN levels (Table 2).

3.2. The Serum Levels of ECP Are Similar among Patients with ALS, AD, and PD and Healthy Controls.
The levels of ECP were not significantly different in the sera from patients with ALS (24.1 ng/mL; SD = 24.5 ng/mL; range, 0.4–88.8 ng/mL), AD (15.4 ng/mL; SD = 17.4 ng/mL; range, 0–97.1 ng/mL), and PD (15.8 ng/mL; SD = 15.2 ng/mL; range, 0–77.8 ng/mL) and healthy controls (21.1 ng/mL; SD = 27.4 ng/mL; range, 1.7–109.9 ng/mL; Figure 1(b), Table 2). Correlations between ECP levels and clinical indicators were also compared. No meaningful correlations were observed between ECP levels and each indicator. Hence, EDN, but not ECP, may serve as an indicator for ALS.

3.3. Prediction of Performance of EDN as an Indicator for ALS.
A receiver operating characteristic (ROC) curve was used to determine the performance of EDN and ECP correlated among ALS, AD, and PD. EDN showed the best performance with 88.53% accuracy, 77.27% sensitivity, and 84.09% specificity when the cut-off concentration was set at 23.43 ng/mL for ALS. ROC curve analysis also showed that EDN had the highest area under the curve (AUC) value of 0.8264. As expected, values for AD and PD were 0.5294 and 0.5538, respectively, indicating a nearly random distribution (Figure 2(a)). For ECP, 54.55% sensitivity, 63.64% specificity, and 69.61% accuracy were detected for predicting ALS. The AUC value for ALS was 0.5754, similar to a random

distribution, and AUC values for AD and PD were 0.5025 and 0.5089, respectively (Figure 2(b)). These results indicate that EDN, but not ECP, may serve as an ALS indicator.

4. Discussion

Damage-associated molecular patterns (DAMPs) play an important role in stimulating macrophages and T lymphocytes [41]. Recent studies have indicated that some DAMPs including ROS [42], HSPs [43], and HMGB1 [43] are present or are overexpressed in the spinal cord [44] and motor cortex in SOD1-G93A transgenic mice and/or patients with ALS [45, 46]. In this study, we report that another DAMP, EDN, was elevated in the sera of patients with ALS. EDN is secreted from human activated eosinophils and neutrophils [47] and is a powerful and important neurotoxin that causes neuronal and axonal damage by inducing loss of normal cell shape [33, 48]. Furthermore, the severe spongy vacuolation of the white matter that is seen in the brainstem, cerebellum, and spinal cord in mice is also caused by EDN overexpression [36, 49]. This phenomenon suggests that the eosinophil-secreted protein EDN plays a crucial role in progressive neurodegenerative disorders. Our analyses showed no significant correlation between the serum concentration of EDN and the stage of ALSFRS-R. ALSFRS-R integrates various aspects of the ALS clinical condition including muscle power, control ability, vigor, and voluntary movement [50, 51]. Our finding may suggest that the neuroinflammation resulting from elevated EDN occurs in the early stage of ALS and does not correlate with disease progression.

We also observed no interaction between the level of EDN and the age of patients with ALS. In the elder population, chronic inflammation increases, and inflammatory stimulation is upregulated during aging [52, 53]. Therefore, we propose that EDN levels correlate with neuroinflammation and may be highly specific to ALS without aging effects. Therefore, EDN may be potentially useful for early diagnosis of ALS caused by peripheral neuroinflammation at any age.

Neuroinflammation is critical in the pathogenesis of ALS [54]. High numbers of dendritic cells and high levels of monocyte chemotactic protein-1 (MCP-1) are found in the ALS mouse model, which shows neuroinflammation [55, 56]. MCP-1 has been implicated as a chemokine that attracts monocytes, T cells, and dendritic cells [57]. MCP-1 may chemoattract various immunocytes to inflammatory sites and induce more severe neuroinflammation [56, 58]. In

TABLE 2: The concentrations of EDN and ECP in sera from patients with ALS, AD, and PD and from controls.

Group (N)	EDN mean (SD, range)	EDN P value					ECP mean (range)	ECP P value				
		Control	AD	PD	ALS			Control	AD	PD	ALS	
Control (44)	21.0 (14.9, 8.6–79.4)	—					21.1 (27.4, 1.7–109.9)	—				
AD (39)	28.3 (36.6, 1.9–158.0)	0.23	—				15.4 (17.4, 0–97.1)	0.26	—			
PD (40)	24.8 (20.7, 3.1–95.5)	0.33	0.61	—			15.8 (15.2, 0–77.8)	0.27	0.73	—		
ALS (44)	45.7 (29.3, 8.9–140.9)	<0.005	0.01	0.02	—		24.1 (24.5, 0.4–88.8)	0.06	0.07	0.06	—	
[b]ALSFRS-R		Control	0–9	10–19	20–29	30–39		Control	0–9	10–19	20–29	30–39
0–9 (15)	42.6 (27.6, 11.9–99.9)	0.63	—				18.7 (19.1, 4.3–77.7)	0.60	—			
10–19 (11)	45.9 (37.5, 8.9–140.9)	0.17	0.24	—			25.6 (27.4, 4.3–78.4)	0.43	0.71	—		
20–29 (7)	39.9 (21.1, 14.6–69.7)	0.08	0.59	0.90	—		21.6 (22.5, 6.3–66.3)	0.20	0.50	0.91	—	
30–39 (7)	51.3 (25.8, 17.9–89.3)	0.27	0.35	0.66	0.49	—	26.2 (27.6, 0.4–77.5)	0.35	0.66	0.17	0.96	—
40–47 (4)	57.4 (37.6, 18.3–108.7)	0.75	0.75	0.33	0.33	0.92 (—)	40.9 (35.4, 12.1–88.8)	0.42	0.75	0.33	0.33	0.42 (—)
[c]Duration		Control	1–12	13–24	25–36	>37		Control	1–12	13–24	25–36	>37
1–12 (31)	50.3 (32.7, 8.9–140.9)	0.83	—				18.9 (20.3, 4.3–77.4)	0.75	—			
13–24 (8)	31.3 (12.2, 16.6–48.6)	0.13	0.27	—			32.5 (31.2, 4.3–88.8)	0.88	0.70	—		
25–36 (3)	49.0 (15.1, 33.2–50.2)	NA	[f]NA	NA	—		58.5 (24.1, 31.5–77.7)	NA	NA	0.33	—	
>37 (2)	27.9 (13.2, 18.6–37.3)	NA	NA	NA	NA	—	18.4 (17.1, 6.3–30.5)	NA	NA	NA	NA	—
[d]Age		Control	41–50	51–60	61–70	71–80		Control	41–50	51–60	61–70	71–80
41–50 (7)	58.9 (42.2, 20.9–140.9)	0.30	—				40.7 (30.6, 10.9–78.4)	0.56	—			
51–60 (19)	35.8 (21.2, 8.9–101.8)	0.06	0.49	—			19.3 (21.1, 4.3–77.7)	0.82	0.56	—		
61–70 (13)	53.5 (30.2, 14.6–108.7)	0.48	0.44	0.91	—		65.4 (26.3, 6.4–88.8)	0.72	0.57	0.93	—	
71–80 (5)	45 (28.1, 16.6–83.4)	0.45	0.95	0.45	0.35	—	9.9 (7.8, 0.4–20)	0.78	0.45	0.68	0.35	—
[e]Onset type		Control	Brainstem	Hand	Foot			Control	Brainstem	Hand	Foot	
Brainstem (13)	42.4 (25.2, 14.6–99.9)	0.89	—				19.8 (21.9, 4.3–77.7)	0.54	—			
Hand (12)	51.1 (30.9, 8.9–108.7)	0.68	0.54	—			22.9 (28.7, 6.2–77.5)	0.90	0.86	—		
Foot (19)	44.7 (32.3, 11.9–140.9)	0.92	0.88	0.62	—		27.7 (20.4, 0.42–88.8)	0.39	0.46	0.39	—	

[a] Concentrations are expressed as ng/mL.
[b] ALSFRS-R is a score from 0 to 48 which assesses disability in patients with motor neuron diseases. 0 means serious, and 48 means normal.
[c] Categories are based on the duration of symptoms (months).
[d] Categories indicate age at time of blood collection (years).
[e] Categories indicate the type of onset.
[f] The NA were divided according to the unanalyzable group (sample number of subgroup <3).
The one-way ANOVA test and Bonferroni multiple comparison test were used to determine the significance among the test groups of control, AD, PD, and ALS.
The Spearman rank correlation test was used to correlate the levels of EDN and ECP versus the subgroups of age, ALSFRS-R, duration, and onset types.

(a)

(b)

FIGURE 1: Serum EDN is elevated in patients with ALS. Serum concentrations of EDN (a) and ECP (b) in controls ($N = 44$) and patients with AD ($N = 39$), PD ($N = 40$), and ALS ($N = 44$). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$. The bold black line indicates the mean.


(a)

(b)

FIGURE 2: The performance of EDN as an indicator for ALS. ROC curves represent the performance of a prediction method. The curves for EDN (a) and ECP (b) for predicting the neurodegenerative diseases AD, PD, and ALS. The area under the curve (AUC) was calculated.

ALS, excessive EDN in hyperimmune conditions may be an autoaggressive factor that interacts with normal neurons to damage those cells and destroy their function, similar to autoimmune diseases [59]. Therefore, higher levels of EDN in ALS may be a damage factor, signal, or immune response. Nevertheless, the mechanisms are still unclear.

Although macrophages, mast cells, and T cells are reported to induce neuroinflammation in the cortex and spinal cord in ALS [60] and although many inflammatory molecules including interleukin-6, interferon-γ, tumor necrosis factor-α, and nitric oxide are elevated in the serum of patients with ALS [61, 62], no studies have elucidated the roles of EDN in ALS. This study is the first to report a correlation between EDN and ALS, and we propose that EDN may participate in the pathogenesis of ALS and may serve as an ALS indicator.

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