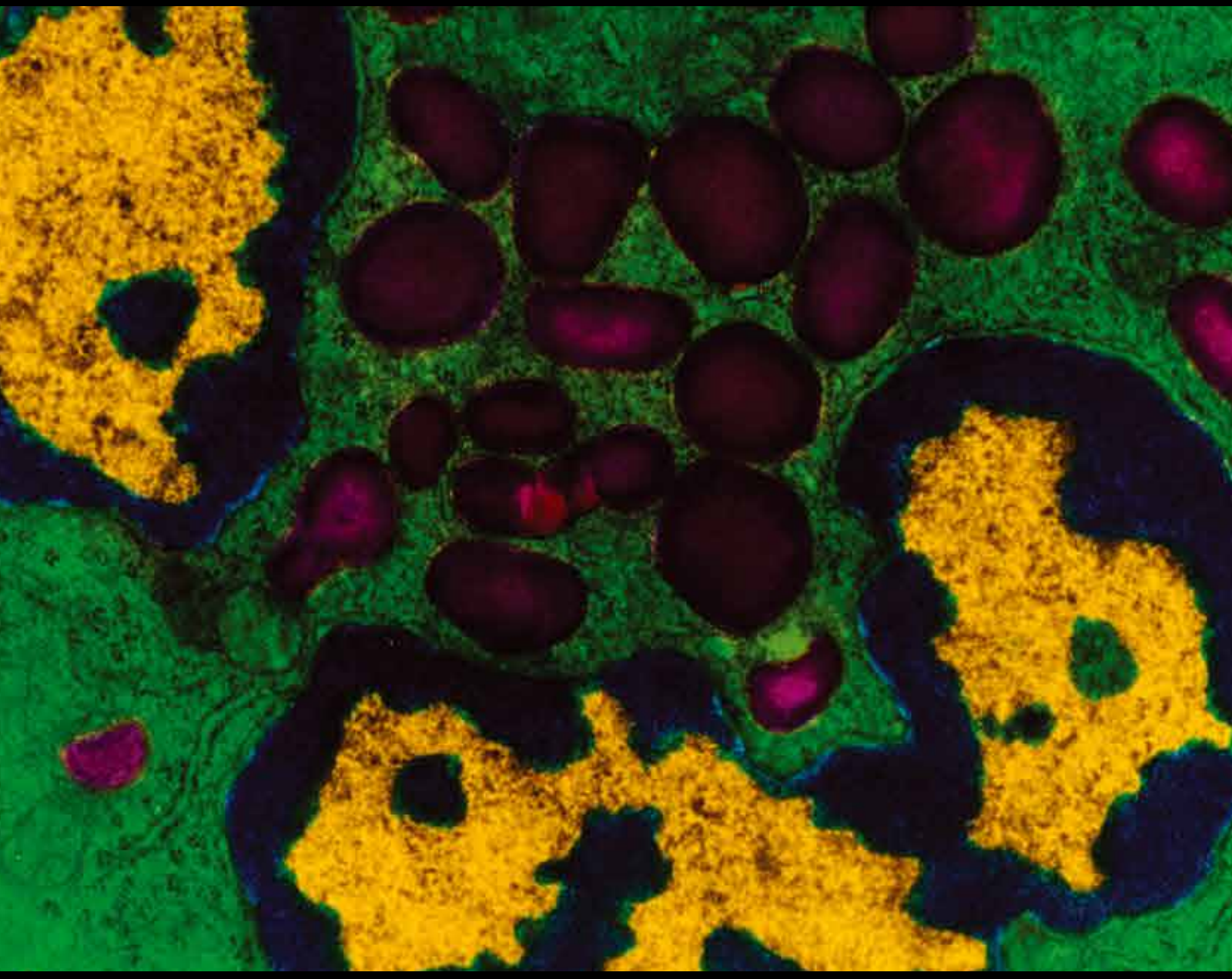
Mediators of Inflammation

The Role of Inflammatory Mediators in Immune-to-Brain Communication during Health and Disease

Guest Editors: Diego Gomez-Nicola, Jessica Teeling, Carmen Guaza, Jonathan P. Godbout, and Dennis D. Taub



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Editorial Board

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Editorial

The Role of Inflammatory Mediators in Immune-to-Brain Communication during Health and Disease

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The field of neuroimmunology is providing growing evidence of active crosstalk between the immune system and the nervous system in health and during diverse pathological conditions. Experimental and clinical research now suggests that signaling from periphery to brain is important for maintaining homeostasis, but also has the potential to impact brain disease initiation or progression. Knowledge and understanding of the cellular and molecular mechanisms governing immune-to-brain bidirectional communication is providing key insights to better model neuroimmune communication, understand the clinical implications, and design better therapies for CNS disorders with an inflammatory component.

This special issue contains 16 papers, representing original research articles, clinical research articles, and reviews, ranging from detailed studies of the key molecular determinants of the neuroinflammatory reaction to the complex neuroimmune component of diverse neurological diseases.

"Systemic immune activation leads to neuroinflammation and sickness behavior in mice" by S. Biesmans et al. describes a study that investigates the effect of systemic LPS administration on the CNS by assessing changes of glial cells, cytokine production, and various behavioural tasks. The use of GFAP-luc mice provides novel insight into the glial response to systemic inflammation. The study also provides insights into the kinetics of behavioural changes in response to systemic inflammation, when measured in the same subject. *"Immune privilege as an intrinsic CNS property: astrocytes protect the CNS against T-cell-mediated neuroinflammation"* by U. Gimsa et al. is a review of the literature on neuroimmune interactions between CNS astrocytes and peripheral T cells that attempt to gain access into the CNS. This review provides evidence of these astrocyte-T cell interactions in both health and disease. The authors discuss mechanisms by which astrocytes regulate T cells to help direct both pro- and anti-inflammatory responses. Overall, the authors review the evidence that astrocytes regulate T cells at multiple check points to help ensure neuroprotection and maintenance of an anti-inflammatory environment within the CNS. *"Activation of protease-activated receptor 2-mediated signaling by mast cell tryptase modulates cytokine production in primary cultured astrocytes"* by X. Zeng et al. is an original article that examined the role of tryptase, which is a protease-activated receptor (PAR)-2 agonist. Tryptase is produced by mast cells and activates the PAR-2 receptor. The authors show evidence that tryptase increased IL-6 and TNF-a production by primary astrocytes. Antagonism of PAR-2 prevented the induction of these cytokines by primary astrocytes. Moreover, the authors outlined two different signaling pathways by which tryptase promotes IL-6 (PI3-kinase dependent) and TNF in astrocytes (P38/JNK-kinase dependent). Taken together this data highlight a way in which peripheral immune cells (i.e., mast cells) can functionally communicate with astrocytes and potentially influence inflammatory responses within the CNS. *"ATP is required and advances cytokine-induced gap junction formation in microglia in vitro"* by P. J. Sáez et al.

reports on the effects of ATP on microglial cells. In this research article the authors use an in vitro approach to study the function of microglial hemichannels and gap junction channels, and their data support the idea that extracellular ATP affects the cellular communication between microglia through autocrine and paracrine mechanisms. *"Possible involvement of TLRs and hemichannels in stress-induced CNS dysfunction via mastocytes, and glia activation"* by A. Aguirre et al. reviews the involvement of a TLR-mediated pathway to control neuroinflammatory responses within the CNS. The authors suggest that mastocytes and glial cells sense the inflammatory tone, via TLR activation, driving the release of inflammatory mediators and further amplification of neural damage.

A significant part of this special issue is devoted to the study of multiple sclerosis, as a paradigm of immune-to-brain communication. *"Persistent inflammation in the CNS during chronic EAE despite local absence of IL-17 production"* by S. F. G. Zorzella-Pezavento et al. describes a study that investigates the role of proinflammatory cytokines in immune infiltrates during the acute and chronic diseases stages of experimental allergic encephalitis (EAE). The results show that the levels of IFN-g are elevated in the periphery and CNS in both the acute and chronic stages of disease. In contrast, IL-17 is only found in the CNS at the acute stages; the authors suggest that this is due to a decline in Treg cells in the CNS at the chronic phase. The study did not investigate the role of myeloid cells, which may be an alternative explanation for persisting infiltrates in the CNS, despite the lack of IL-17. *"Local overexpression of interleukin-11 in the central nervous system limits demyelination and enhances remyelination"* by A. Maheshwari et al. provides evidence that IL-11 promotes remyelination in the cuprizone model of demyelination associated with reduced microglial activation. It remains to know whether the effect of IL-11 on myelination is direct or indirect via microglia/macrophages. *"Growth arrest specific gene 6 protein concentration in cerebrospinal fluid correlates with relapse severity in multiple sclerosis"* by P. P. Sainaghi et al. reports a clinical study that shows a correlation of Gas-6 in CSF with the severity of relapse in relapsing-remitting multiple sclerosis patients. However, the Gas-6 levels in CSF were not predictive for the long-term outcomes. *"Regulation of immune cell infiltration into the CNS by regional neural inputs explained by the gate theory"* by Y. Arima et al. reviews the modes of infiltration of immune cells into the CNS parenchyma. In particular, the authors propose the "gate theory" as an alternative explanation for the control of cell extravasation into the CNS in pathological conditions such as multiple sclerosis.

Some articles focus on the study of the neuroimmune component of neurodegenerative diseases such as Alzheimer's or Parkinson's, or other brain diseases. *"Role of scavenger receptors in glia-mediated neuroinflammatory response associated with Alzheimer's disease"* by F. Cornejo and R. von Bernhardi is a detailed review about the inflammatory mechanisms mediated by microglia and astroglia on the pathological events in the Alzheimer's disease, emphasizing the relevance of A scavenger receptors for Ab clearance. *"Role of neuroinflammation in adult neurogenesis and Alzheimer disease: therapeutic approaches"* by A. Fuster-Matanzo et al. reviews the influence of the neuroinflammatory component of Alzheimer's disease over the regulation of adult neurogenesis, aiming at summarizing the reported effects of several inflammatory mediators and finding new avenues into potential therapeutic interventions. *"Cellular and molecular mediators of neuroinflammation in the pathogenesis of Parkinson's disease"* by S. V. More et al. reviews the current evidence supporting a detrimental role of microglial cells during the pathogenesis of Parkinson's disease. Current evidence from human studies highlights the neurotoxic activity of diverse inflammatory mediators or infiltrated immune cells in Parkinson's disease, although further experimental evidence is needed to fully understand the neuroinflammatory reaction in this pathology. *"MMP-3 contributes to nigrostriatal dopaminergic neuronal loss, BBB damage, and neuroinflammation in an MPTP mouse model of Parkinson's disease"* by Y. C. Chung et al. is an original research article that examined the role of metalloproteases 3 (MMP3) in a mouse model of Parkinson disease. These authors provide novel evidence that matrix MMP3 plays a key role in increased leukocyte infiltration and dopaminergic cell loss after MPTP. For instance, MMP3 knockout mice had attenuated damage to the blood brain barrier (BBB), reduced infiltration of ED-1+ and CD3+ leukocytes, and reduced neuronal loss. Moreover, motor function was maintained in the MMP3 knockout mice after MPTP injection compared to the wildtype MPTP-injected mice. Thus, MMP3-mediated breakdown of the BBB and enhanced leukocyte infiltration into the brain play a significant role in nigrostriatal dopaminergic neuronal loss after MPTP. *"Are onconeural antibodies a clinical phenomenology in paraneoplastic limbic encephalitis?"* by H. Zhang et al. is a detailed review about the role of brain-specific antibodies in neurological diseases and in particular those associated with peripheral malignancies. *"The causative pathogen determines the inflammatory profile in cerebrospinal fluid and outcome in patients with bacterial meningitis"* by D. Grandgirad et al. is a clinical study that examined cerebrospinal fluid (CSF) from patients with meningitis caused by one of three different pathogens (*Streptococcus pneumonia, Neisseria mengitidis*, or *Haemophilus influenza*). The authors' findings indicate that pneumococcal meningitis was associated with high levels of inflammatory mediators in the CSF including IFN-γ, MCP-1, and MM9. Furthermore, the higher rates of fatal outcome in patients with pneumococcal meningitis were associated with correspondingly high levels of proinflammatory cytokines in the CSF including TNF-α, IL-1β, and IL-6. Overall, patients with pneumococcal meningitis had an elevated CNS inflammatory response and higher mortality rate than patients with either *Neisseria mengitidis* or *Haemophilus influenza*. *"Prognostic value of inflammatory mediators in 1-year outcome of acute ischemic stroke with middle cerebral artery stenosis"* by X. Gong et al. is a clinical study reporting the prognostic value of diverse inflammatory mediators in ischemic stroke. Elevated hs-CRP was found to predict 1-year poor outcome in acute stroke. The combination of increased hs-CRP, WBC, or HCY had a stronger predictive value in poor outcome than individual elevated mediator.

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Review Article

Immune Privilege as an Intrinsic CNS Property: Astrocytes Protect the CNS against T-Cell-Mediated Neuroinflammation

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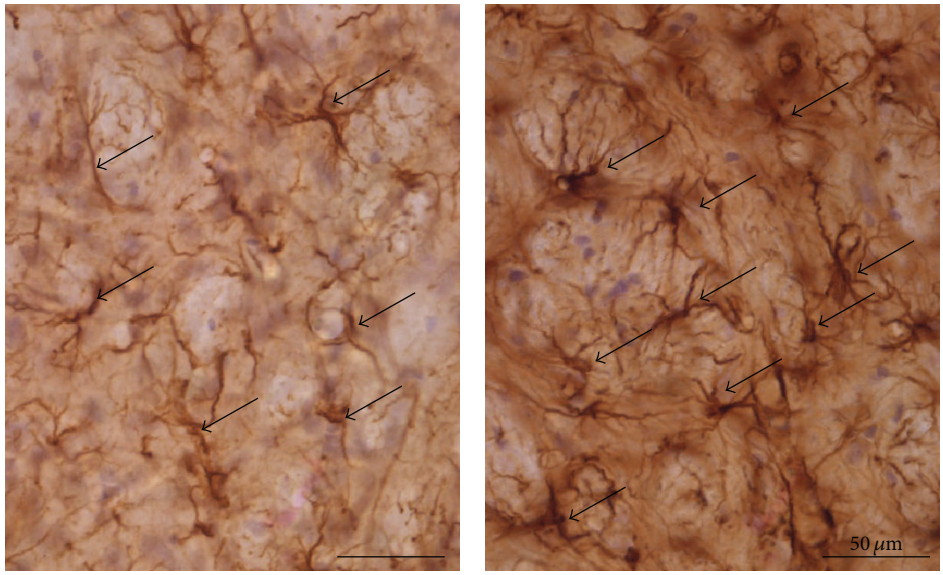
Astrocytes have many functions in the central nervous system (CNS). They support differentiation and homeostasis of neurons and influence synaptic activity. They are responsible for formation of the blood-brain barrier (BBB) and make up the glia limitans. Here, we review their contribution to neuroimmune interactions and in particular to those induced by the invasion of activated T cells. We discuss the mechanisms by which astrocytes regulate pro- and anti-inflammatory aspects of T-cell responses within the CNS. Depending on the microenvironment, they may become potent antigen-presenting cells for T cells and they may contribute to inflammatory processes. They are also able to abrogate or reprogram T-cell responses by inducing apoptosis or secreting inhibitory mediators. We consider apparently contradictory functions of astrocytes in health and disease, particularly in their interaction with lymphocytes, which may either aggravate or suppress neuroinflammation.

1. Introduction

Within the central nervous system (CNS), astrocytes are the most abundant cells. Their main task is to maintain the physiological homeostasis of neurons by providing a stable microenvironment and growth factors. Astrocytes form multicellular syncytia *in vivo* that ensure neuronal homeostasis by taking up excess neurotransmitters and buffering the ionic content of the extracellular medium in the brain. Astrocyte membranes contain numerous neurotransmitter receptors and transporters and can therefore sense and regulate formation, stability, and efficacy of synapses [1]. Recently, they have been shown to play a role in synaptic activity and regulating neuronal circuitry [2–4].

Astrocytes are dysfunctional in various neurological disorders such as epilepsy, amyotrophic lateral sclerosis, hepatic encephalopathy, stroke, and focal cerebral ischaemia (reviewed in [5]). Dysfunction is often accompanied by astrocytic hypertrophy and an increased number of astrocytic processes, termed astrogliosis [6]. Astrocytes also show these signs of activation in Alzheimer's disease [7, 8] and in Parkinson's disease [9] as well as in its rat model (Figure 1) [10]. Massive astrogliosis has been observed in postmortem tissue of Parkinsonian patients [9, 11–13]. These tissues demonstrated a lack of astrocyte-derived neurotrophins compared to control brains [14, 15]. Because astrocytes support and protect dopaminergic neurons *in vitro* [16], a functional failure of astrocytes may contribute to CNS pathology.

The potential for antigen presentation and production of proinflammatory cytokines by astrocytes has been studied in the neuroinflammatory disease multiple sclerosis (MS) and its animal model experimental autoimmune encephalomyelitis (EAE). They can protect against neuroinflammation by T cells invading the CNS. Thus, they contribute to the immune privilege of the CNS. The privilege is not simply the absence of immune reactions but rather a complicated network of passive and active barriers and of brain tissue. It can modify immune reactions in the CNS so as to minimize the danger



(a) (b)

Figure 1: Astrocytes activated in a rat model of Parkinson's disease. Astrocytes (arrows) in the globus pallidus of rats after unilateral striatallesion of dopaminergic neurons by injection of 6-hydroxydopamine (6-OHDA). (a) Contralateral hemisphere; astrocytes have short cellular processes. (b) Ipsilateral hemisphere; astrocytes are in an activated state characterised by long cellular processes and enlarged cell bodies with an intense staining. Staining of glial fibrillary acidic protein (GFAP). For detailed information, see [10].

of destructive side effects in a tissue with limited ability to regenerate [17]. In this review, we focus on astrocyte functions in health and disease, particularly on their interaction with lymphocytes.

2. Functions of Astrocytes at the Blood-Brain Barrier (BBB)

The BBB limits exchange of solutes between capillaries and the brain parenchyma. Brain capillaries are about 50 to 100 times tighter than peripheral capillaries. This is achieved by complex tight junctions. Astrocytes influence tightness of the BBB by soluble factors that affect endothelial cells [18]. The perivascular space is separated from the brain parenchyma by the basement membrane and the glia limitans, made up of astrocytic end-feet, reviewed in [19]. Notably, it is not the direct contact of astrocytic end-feet with endothelial cells that induces the tightness but soluble factors secreted by them. The presence of numerous astrocytic end-feet close to the BBB allows for a rapid regulation of BBB permeability. Humoural agents that are able to increase BBB permeability and may be secreted by astrocytes include endothelin-1, glutamate, interleukin- (IL-) 1β, IL-6, tumour necrosis factor (TNF), macrophage inflammatory protein- (MIP-) 2, and nitric oxide [20]. Soluble astrocytic factors that induce tight junction formation at the BBB are less well characterized. A recent study has shown that sonic hedgehog, a member of the hedgehog signalling pathway family, is produced by astrocytes. Sonic hedgehog promotes BBB formation and integrity, and hedgehog-mediated signals induce immune quiescence in the CNS [21]. Thus, inhibition of hedgehog signalling exacerbates EAE by increasing demyelination, accumulation of leukocytes in the CNS, and production of interferon- (IFN-) γ and IL-17 by infiltrating T cells [21].

3. Pro- and Anti-Inflammatory Mediators Produced by Astrocytes

Astrocytes are capable of producing a range of proinflammatory cytokines that have been found in the brain of Alzheimer's disease patients such as IL-1α, IL-1β, IL-6, and TNF [22]. It has been shown that amyloid-β_{25-35} in combination with bacterial cell wall lipopolysaccharide (LPS) induced a strong astrocytic production of IL-6 and TNF while neither of the substances alone did [23]. Others found that LPS induced the production of TNF, IL-6, and IL-1 in microglia but not in astrocytes while astrocytes responded neither to LPS nor TNF but to IL-1β by producing TNF and IL-6 [24]. This indicates that astrocytes may be regulated by microglial IL-1β. Microglial cells produce free radicals and proinflammatory cytokines such as TNF-α when exposed to amyloid-β_{1-42} [25, 26]. TNF and superoxide anion production by macrophages cocultured with amyloid-β_{1-42} was strongly reduced in the presence of primary human astrocytes or astrocytoma cells. Interestingly, astrocytes bound amyloid-β_{1-42} and showed activation of the transcription factor NFκB in that study, but unlike in macrophages this activation did not result in TNF production. This indicates that distinct signal transduction pathways are activated in macrophages and astrocytes by inflammation [27]. Indeed, astrocytes can also downregulate microglial activation by the secretion of anti-inflammatory substances such as transforming growth

factor- (TGF-) β and prostaglandin E_2 (PGE_2) [28, 29] and may thereby limit inflammation-induced neurodegeneration. However, activated microglia can also reduce amyloid-β accumulation by phagocytosing and degrading it [30]. Thus, the clinical relevance of both astrocytic and microglial activation has not yet been fully elucidated.

Glia maturation factor (GMF) is produced by astrocytes. It is not only necessary for the growth and maturation of neurons and glia cells, but can also induce the production of proinflammatory cytokines. Overexpression of GMF in astrocytes induces the production and secretion of granulocyte-macrophage-colony stimulating factor (GM-CSF), an activation of microglia and the expression of proinflammatory genes including major histocompatibility complex- (MHC-) II, IL-1β, and MIP-1β [31]. Knockdown of GMF reduces the production of the proinflammatory cytokines and chemokines responsible for EAE [32, 33]. Interestingly, it also inhibits growth of glioblastoma cells by inducing G0/G1 cell cycle arrest *in vitro* [34, 35]. In the brain of Alzheimer's disease patients, GMF is upregulated [36, 37]. However, what drives astrocytes to upregulate GMF to a level where it contributes to tissue damage is unknown.

Astrocytes produce or take up, store, and reexocytose a range of neurotrophins neuroprotective in EAE [38–41], dementia of the Alzheimer type [42], and Parkinson's disease [43, 44]. Astrocytes are the major source of nerve growth factor (NGF) and glial cell line-derived neurotrophic factor (GDNF) in the CNS [45–47]. In brain tissue of Parkinson's disease patients, GDNF, NGF, and brain-derived neurotrophic factor (BDNF) are deficient [14, 15], hence the clinical trials of therapeutic GDNF injection into the brain of Parkinson's patients. While intraputaminal infusion of GDNF was safe and improved motor functions in a small group of patients over one [48] and two years [49], a randomized placebo-controlled trial found that motor function has not improved [50]. Notably, from all the 32 genes associated with astrocyte function described in this review, only GDNF was found to be associated with a disease: "major depressive disorder." For this, see the NCBI catalog of genomewide association studies (GWAS) (http://www.genome.gov/gwastudies/).

On the other hand, as mentioned above, astrocytes are a major source of the proinflammatory cytokines IL-1β and IL-6 in the brain [51, 52]. Transgenic mice that lack IL-6 production are resistant to EAE induction [53, 54]. This is due to a blockade of activation and differentiation of autoreactive T cells in the periphery with both T helper (Th) 1 and Th2 cells differentiation being affected [53]. Very recently, dendritic cells have been identified as a sufficient and probably the main source for EAE induction [55]. Whether astrocytic IL-6 plays a decisive role in the etiogenesis of EAE has been ruled out in animal models. Transgenic mice that overexpress IL-6 in astrocytes but are otherwise deficient in IL-6 develop a mild form of ataxia, but no symptoms of lymphocyte-driven EAE. These mice had indeed cellular infiltrates in the cerebellum independent of MOG immunisation [56]. Thus, the observed ataxia may be a result of a general inflammatory process in the brain.

It is known that IL-1β plays an important role in MS and EAE. Families with a high IL-1β over IL-1 receptor antagonist (IL-1Ra) production ratio have a higher risk to have a patient relative with MS than families with a low ratio [57]. Mice deficient in IL-1 receptor type I (IL-1RI−/−) are resistant to EAE induction [58, 59]. Apparently, IL-1β is necessary for the induction of IL-17-producing T cells (Th17) [59]. IL-17 has been shown to be crucial for the development of EAE [60, 61]. However, both IL-6 and IL-1β do not necessarily have only detrimental effects. Recently, IL-6 has been demonstrated to induce IL-10 in T cells and thus inhibit proinflammatory responses of Th1 cells [62]. The production of IL-1β and IL-6 does not necessarily lead to neuronal damage because these cytokines also induce upregulation of Fas ligand (FasL) in astrocytes, which may induce T-cell apoptosis [63] (see below). In addition, IL-1β and IL-6 are messengers between the brain, particularly the hypothalamic-pituitary-adrenal axis, and the immune system. Thus, IL-1β produced during EAE upregulates glucocorticoid production which has a downregulatory effect on inflammation [64].

4. Interactions of Astrocytes and T Lymphocytes

4.1. Induction of Apoptosis in Activated T Cells. Activated T cells can cross the BBB not only in neuroinflammatory diseases but also in the healthy brain [65, 66]. Later, it has been shown that in macrophage-depleted mice, activated T cells which extravasate are not able to enter the brain parenchyma via the basement membrane but accumulate in the perivascular spaces [67]. Matrix metalloproteinases (MMP-) 2 and -9 are necessary to cross the basement membrane after local digestion [68]. These enzymes could be produced by perivascular macrophages.

These infiltrating T cells may combat infection, but damage to tissue needs to be avoided, and in particular that mediated by Th1 and cytotoxic T cells and accompanied by inflammation. Inflammatory cytokines such as TNF-α are neurotoxic. Given that neurons have a very limited capacity to regenerate in the mature brain, side effects could be detrimental. One mechanism preventing damage is elimination of T cells: astrocytes induce apoptosis in these cells [69–71]. This effect is mediated by the expression of FasL (CD95L) by astrocytes [63, 72, 73]. In EAE, FasL expressing astrocytes exist in close vicinity to apoptotic T cells [74, 75]. The same mechanism of enforcing immune-privilege has been observed in placenta [76–79], testes [80], and anterior chamber of the eye [81]. A downside of this mechanism is that astrocytoma express FasL and thus escape immune attack [82, 83].

4.2. Astrocytes as Antigen-Presenting Cells in Neuroinflammation. In neuroinflammation, astrocytes can act as antigen-presenting cells (APCs) [84, 85]. While microglia express MHC-II readily upon activation *in vivo* and *in vitro*, astrocyte MHC-II expression occurs only during prolonged inflammation *in vivo* [86] or *in vitro* under stimulation by interferon- (IFN-) γ [87]. This MHC-II induction may be suppressed by neurons via a mechanism that has not fully been elucidated. One study claims that cell-cell contact is required [88] while another one found that secreted glutamate and norepinephrine could inhibit IFN-γ induced MHC-II expression

in astrocytes [89]. In keeping with this, neuronal loss induces MHC-II expression in astrocytes [88, 90], supporting the view that astrocytes can present antigen only during severe neuroinflammation. The expression of costimulatory B7 molecules by astrocytes both *in vivo* and *in vitro* has been controversially discussed. While some authors found B7 expression on astrocytes [91–94], others did not [95, 96]. Functioning as APCs *in vitro*, astrocytes have been found to stimulate differentiated T cells; and interestingly, they stimulate Th2 cells more efficiently than Th1 cells [87, 97]. Th2 cells may be less damaging than the cellular immune responses, and hence the preferred agents of protection against infection in the CNS. Thus, astrocytes from transgenic mice expressing MS-associated MHC-II human haplotypes HLA-DR2 and HLA-DR4 induced a mixed Th1/Th2 cytokine response in MOG-specific T cells, whereas dendritic cells induced a Th1 response [98]. One can only speculate about the biological relevance of an astrocyte-mediated Th2 bias. In EAE, T cells typically enter the CNS as activated, differentiated Th1 cells. However, the T-cell population may not consist exclusively of Th1 cells. If astrocytes preferentially restimulate Th2 cells [87, 97], the proportion of these cells could increase, thus favouring an anti-inflammatory microenvironment. Also, memory T cells are recruited to the CNS during EAE [99]. Memory cells are heterogeneous and part of the population is not biased for a certain Th subpopulation, yet. Thus, it is tempting to speculate that astrocytes may prevent induction of a Th1 cytokine profile of memory cells in the CNS [100]. The astrocyte-mediated bias towards Th2 responses cannot be explained by their cytokine secretion as astrocytes do not produce IL-4, which is the main inductor of Th2 responses, but might rather reflect the signal strength of the MHC-II-T-cell receptor (TCR) interaction. Lowering the signal strength has been found to favour Th2 differentiation [101]. For instance, the surface density of MHC-II expression determines the cytokine profile of T cells with low MHC-II expression levels favouring Th2 responses [102]. Astrocytes do not readily express MHC-II molecules and are thus likely to deliver a weaker TCR signal than "professional" APCs with higher density of MHC-II molecules on their surface.

4.3. Suppression of T-Cell Functions. In EAE, infiltrating T cells do not proliferate in the target organ [103]; this has been ascribed to the influence of astrocytes [104]. *In vitro*, astrocytes can either suppress [105–107] or stimulate [87, 97, 108] T-cell functions. In coculture studies, astrocytes induce hyporesponsiveness in T cells. This was interpreted as a result of downregulation of the TCR [105] and insufficient stimulation by low levels of ICAM-1 on astrocytes [106]; this would limit adhesion of T cells to astrocytes, so that the two cells ignore each other. As this would not silence invading T cells in the CNS, other mechanisms may have been involved which are not fully understood, yet.

T-cell activation is tightly regulated by surface molecules, providing scope for immunotherapy [109–111]. While the primary costimulatory molecule CD28 and its homologue CTLA-4 (cytotoxic T-lymphocyte-associated antigen-4, CD152) on T cells engage the same ligands B7-1 (CD80) and B7-2 (CD86) on APCs, CTLA-4 binds with 10–100-fold higher affinity than CD28 [110, 112]. CD28 signaling initiates, sustains, and enhances T-cell activation while CTLA-4 signaling inhibits T-cell activation and attenuates ongoing responses [110, 113, 114]. The relevance of this has been demonstrated by genetic inactivation of CTLA-4 in mice, which leads to lymphoproliferative disease and early death [110, 112]. T cells of this mouse strain proliferate spontaneously *ex vivo* and show an activated phenotype stressing the central role of CTLA-4 in attenuating unwanted T-cell responses. In contrast to CD28, which is constitutively expressed on the surface of T cells, CTLA-4 is not detectable on resting T cells [114]. Expression of CTLA-4 mRNA and CTLA-4 protein on the T-cell surface is induced upon activation. CTLA-4 is stored intracellularly, and its surface expression is strictly controlled with a peak after 48 h–72 h after T-cell stimulation [114, 115]. Blockade of CTLA-4 in mouse models of autoimmune diseases increases the incidence of EAE [111, 116]. Short blockade of CTLA-4 during priming of the immune response has lasting effects, suggesting that failure in the regulation of CTLA-4 would have long-lasting impact on immune responses including autoimmunity [117]. Thus, giving agonistic CTLA-4 signals might be a promising strategy for controlling inflammatory responses in the CNS, particularly as CTLA-4 is highly expressed on the T cells which accumulate there [118].

Our own study showed that astrocytes inhibit T-cell proliferation, production of IL-2 and IL-10, and expression of the IL2R α-chain (CD25) [107]. Functionally, astrocytes mediated these effects by upregulating CTLA-4 on Th1 and Th2 cells. Although inhibition did not require astrocyte contact with T cells, the mechanism was independent of the major inhibitory cytokine TGF-β. The study provided optimal stimulation for T cells by having professional APCs and antigen in the cultures when astrocytes were added. Thus, astrocytic inhibitory or stimulatory effects could be discerned from baseline effects occurring during T cell-APC interaction. In this way, we also avoided differences in the stimulatory capacity of astrocytes towards Th1 versus Th2 cells [87, 97]. The interpretation is supported by a recent study showing that astrocytes inhibited proliferation and IFN-γ, interleukin-(IL-) 4, IL-17, and TGF-β secretion levels of encephalitic T cells *in vitro* unless they were pretreated with IFN-γ. They even promoted T-cell proliferation, presumably by additional antigen presentation [119]. The inhibitory effect of astrocytes could be ameliorated by IL-27 neutralisation [119]. IL-27 has been shown to suppress Th17 cells and thereby EAE [120, 121]. Also, it negatively regulates Th17 cells during chronic inflammation of the CNS resulting from chronic infection with *Toxoplasma gondii* [122]. Coculture of astrocytoma cell lines with CD3/CD28-activated T cells revealed suppression of T-cell proliferation. The effect was more pronounced when direct contact was allowed between astrocytes and T cells but remained strong when astrocytes and T cells were separated by cell culture inserts [123]. The finding that T-cell proliferation was still inhibited by astrocytes when astrocytes and T cells were separated by a cell culture insert or a transwell-membrane showed that a soluble factor produced by astrocytes is responsible for this inhibition [107, 123, 124].

However, astrocytes might conceivably have protruded cellular nanotubes through the cell culture inserts so as to contact the T cells. The separating membranes had pore sizes of 200 nm [123] or 400 nm [107, 124]. An electron-microscopical study of astrocytes growing on engineered surfaces showed that astrocytes extend nanotubes with a diameter below 100 nm to make contact with other cells and may even exchange substances via these nanotubes [125]. This may be a mechanism which allowed astrocytes to contact the T cells physically. Cell-cell contact did not bear sole responsibility for the control of T-cell proliferation, since astrocyte-conditioned supernatant also inhibited T-cell proliferation [124]. Despite being of interest for immunotherapy, the nature of this soluble inhibitory factor remains unclear. Blockade of TGF-β had no [124] or only a minor effect [107] on the inhibition of T-cell proliferation. Inhibition of nitric oxide production also did not reverse the inhibitory effect [123, 124]. Furthermore, inhibition of indoleamine-2,3 dioxygenase (IDO) by methyltryptophan did not affect astrocyte-mediated inhibition of T-cell proliferation [123].

IDO is a tryptophan-degrading enzyme and as such inhibits T-cell proliferation. It has been proposed as a major player in the immune privilege of the placenta [126]. Astrocytes and microglia are capable of expressing IDO *in vitro* and *in vivo* upon activation with IFN-γ [127]. IDO blockade in EAE mediates disease exacerbation, suggesting that IDO induction by Th1-derived IFN-γ may play a role in self-limiting autoimmune inflammation during EAE and MS [128]. IDO can also induce tolerance of tumours in the CNS [129]. PGE_2 induces IDO in dendritic cells [130, 131]. Systemic administration of cytosine-phosphate-guanine dinucleotide (CpG), a frequent dinucleotide in bacterial DNA and therefore detected by pattern recognition receptor Toll-like receptor-9 (TLR-9), upregulates IDO in plasmacytoid dendritic cells, where it is required for activation of regulatory T cells (Tregs), and blocks their conversion into Th17 cells [132]. Although likely, whether IDO induction in astrocytes by PGE_2 or CpG plays a role in the CNS and whether astrocytes can induce Treg activation is one of the open questions concerning astrocytes so far. IDO-deficient mice develop exacerbated EAE with enhanced Th1 and Th17 responses [133]. In this model, not only tryptophan depletion was responsible for the effect on T cells but also a downstream tryptophan metabolite from the kynurenine pathway, 3-hydroxyanthranilic acid (3-HAA), was. The kynurenine pathway starts with tryptophan degradation by IDO or tryptophan-2,3 dioxygenase (TDO) leading to 3-HAA. 3-HAA was shown to increase the percentage of Tregs and inhibited Th1 and Th17 cells leading to EAE amelioration [133]. 3-HAA has been shown to be neuroprotective in cytokine-mediated inflammation *in vitro* [134] while other metabolites of the kynurenine pathway such as 3-hydroxykynurenine and quinolinic acid (QUIN) appear to be neurotoxic [135]. Another metabolite of the IDO-kynurenine pathway is kynurenic acid (KYNA) which has been shown to be neuroprotective [136]. Interestingly, activated human astrocytes have been shown to produce large amounts of KYNA but almost no QUIN [137]. Thus, astrocytic IDO activation may lead to various effects which are mostly beneficial.

Astrocytes in a rat EAE model could induce development of Tregs, as has been shown in a study where T cells that had been cocultured with astrocytes not only lost ability to proliferate and inhibit proliferation of antigen-stimulated T cells but also markedly alleviated the disease [138]. Also in this study a heat-sensitive soluble factor was implicated, other than IL-10 or TGF-β [138].

Another surface molecule, B7-H1 (PD-L1), might downregulate T-cell responses in the CNS; it is a member of the B7-family known to downmodulate T-cell activity [139]. In a model of fiber tract injury in the hippocampus of adult mice, it is strongly upregulated on astrocytes while T-cell recruitment to the site of injury was not accompanied by autoimmune demyelination [140].

4.4. Astrocyte-Released Signals That May Influence T-Cell Influx. Astrocytes are efficiently activated by the IFN-γ produced by Th1 cells (see above). Under the influence of IFN-γ, astrocytoma cells upregulate expression of chemokines including CCL3, CCL5, CXCL8, and CXCL10, as well as proinflammatory cytokines such as IL-6 and IL-1β (but also an anti-inflammatory IL-1 receptor antagonist) [123]. Most of these chemokines attract Th1 cells more than Th2 cells, thus aggravating neuroinflammation. Thus, astrocytes may inhibit and delay neuroinflammation, but in case of sustained inflammation accompanied by high IFN-γ levels, they may switch to become potent APCs and even promotors of inflammation [119].

4.5. T-Cell-Mediated Induction of Nerve Growth Factor. Nerve growth factor (NGF) is a member of the neurotrophin family. Growth, differentiation, survival, and maintenance of peripheral and central neurons are facilitated by NGF [143]. NGF administered intracerebroventricularly into marmosets delays the onset of EAE and reduces lesion formation [41]. Subsequent to induction of EAE, mice treated with NGF by intraperitoneal injection exhibited a delayed onset of disease in combination with lower clinical disease scores [144]. Moreover, myelin basic protein- (MBP-) specific T cells retrovirally transduced to secrete high levels of NGF are unable to mediate clinical EAE and suppress induction of EAE by nontransduced MBP-specific T cells in rats [40]. Astrocyte-T-cell interaction results in increased NGF production by astrocytes. This upregulation was found to be dependent on antigen recognition as blockade of MHC-II abrogated the effect, and resting astrocytes which were not able to present antigens did not show an upregulation of NGF production. Neutralisation of the cytokines IFN-γ, IL-4, and IL-10 produced in the cocultures did not affect NGF production [142]. This finding suggests a neuroprotective role of astrocytes during T-cell-mediated inflammation in the CNS. Conversely, cells of the immune system carry NGF receptors, and NGF signalling modulates immune function. Perivascular infiltrates of NGF-treated marmosets decrease IFN-γ and increase IL-10 expression [145]. NGF inhibits the MHC-II inducibility of microglia, thereby limiting antigen-presentation in the CNS [146].

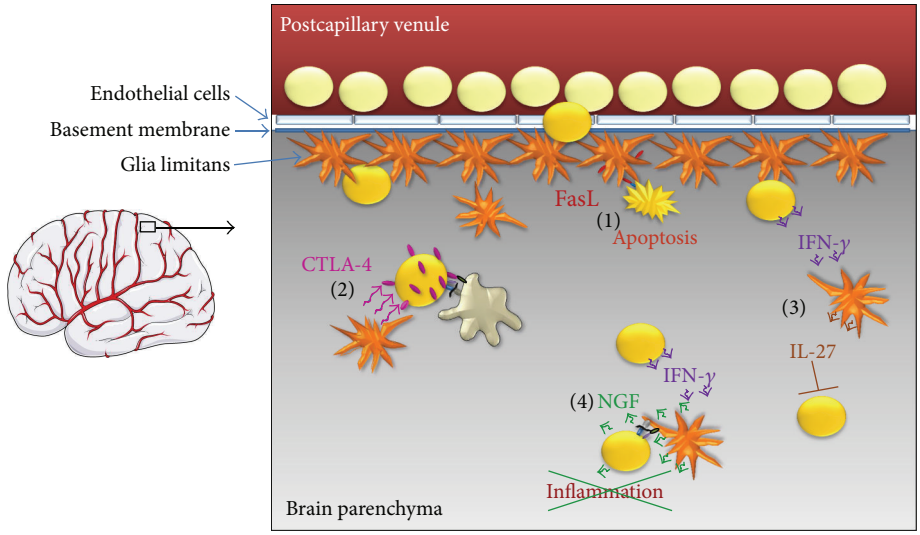


Figure 2: Astrocytes enforce the immune privilege of the CNS (left) at multiple checkpoints employing various mechanisms (right). Astrocytes in the glia limitans are responsible for the exceptional tightness of endothelial tight junctions by producing soluble factors [18]. Despite the BBB, activated T cells (yellow) are able to enter the brain parenchyma (grey) [65]. (1) At the same time, astrocytes in the glia limitans and in the parenchyma may express FasL while activated T cells may express Fas [63, 72, 73]. The ligation of Fas and FasL induces apoptosis of T cells [71]. (2) As this does not fully eradicate infiltrating T cells, the surviving T cells may be restimulated by activated microglia presenting CNS-specific antigens on MHC-II. In the presence of astrocytes, T cells upregulate CTLA-4 [107] which upon ligation of B7 molecules induces a stop of proliferation and anergy of the T cells. (3) IFN-γ produced by invading T cells stimulates astrocytic IL-27 production which suppresses Th17 cells [120, 121, 141]. (4) During sustained T-cell-mediated inflammation, IFN-γ secreted by T cells activates astrocytes to gain the ability to present antigen on MHC-II and costimulate T cells. While this cognate interaction may exacerbate neuroinflammation, it simultaneously leads to an upregulation of NGF production that counteracts neuroinflammation [142]. Also, astrocytes acting as APCs appear to promote Th2 responses and the formation of regulatory T cells [138]. Astrocytes: orange cells; pink: effects leading to CTLA-4 upregulation; green: effects of NGF; dark red: blood; grey: brain parenchyma.

Mechanisms by which astrocytes maintain immune privilege or limit inflammation-induced damage are summarised in Figure 2.

5. Conclusions

For a long time, the CNS has been considered immune-privileged. However, the initial explanation of a strictly sealed BBB weakened when activated T cells were found to cross the BBB in the healthy brain. Clearly, various cells contribute to the phenomenon, including astrocytes, the most abundant cells of the CNS. Astrocytes mediate neuronal differentiation and homeostasis, and evidence is increasing that astrocytes interact with the immune system. The concept of immune privilege of the CNS may be weakening, but it is clear that astrocytes dampen inflammation and have beneficial, neuroprotective effects on the healthy brain. Astrocytes need activation by IFN-γ to unfold their anti-inflammatory potential, in forms such as IL-27 production [141]. Even when unable to prevent T-cell responses in the brain after prolonged provocation (e.g., by IFN-γ), their function does not become purely detrimental. When activated, astrocytes harbour mechanisms of damage limitation, such as production of neuroprotective NGF and preferential restimulation of Th2 over Th1 cells. When this is not sufficient to prevent autoimmune damage to the CNS, it may still control tissue damage to some extent. The overall picture of astrocytes is as CNS-intrinsic cells that combat local inflammation and maintain immune privilege, thus minimising damage.

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Clinical Study

Prognostic Value of Inflammatory Mediators in 1-Year Outcome of Acute Ischemic Stroke with Middle Cerebral Artery Stenosis

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Background and Purpose. Inflammation exists in inception, progression, and reperfusion of acute ischemic stroke. Insightful understanding of correlation in inflammatory mediators and stroke severity with intracranial artery stenosis may improve rational stroke therapy. *Methods*. We prospectively recruited 977 patients with acute noncardioembolic ischemic stroke with MCA stenosis by MRA as none to mild (<50%), moderate (50–69%), severe (70–99%), or occlusive (100%). The peripheral levels of WBC, homocysteine (HCY), and high sensitivity C-reactive protein (hs-CRP) were recorded. All patients were assessed of 1-year outcome by mRS as favorable (0–2) or poor (3–6). *Results*. The levels of WBC, HCY, and hs-CRP had no significant differences in patients with categorized MCA stenosis (all $P > 0.05$). Higher levels of WBC, HCY, and hs-CRP were found in patients with 1-year poor outcome (all $P < 0.05$), but only hs-CRP is an independent predictor (OR 1.06, 95% CI 1.027–1.093, $P = 0.0003$). The combination of any two of increased hs-CRP (>3 mg/L), WBC (>6.91 × 10^9/L), and HCY (>15 μmol/L) had higher power in predicting 1-year poor outcome than the single elevated mediator. *Conclusions*. Elevated hs-CRP independently predicts 1-year poor outcome in acute stroke. The combination of increased hs-CRP, WBC, or HCY had a stronger predictive value in poor outcome than individual elevated mediator.

1. Introduction

Inflammation potentially contributes to destruction of cerebral tissue during the stage of acute ischemic stroke. Originally, inflammation acts as the fundamental part in the process of atherosclerosis [1–4] which is the most common cause of ischemic stroke by arterial thrombosis or embolism. Subsequently, focal acute ischemia will trigger a series of inflammatory cascades which are different from atherosclerotic progression, causing more damage to ischemic cerebral tissues [5]. Moreover, even in the phase of ischemia-reperfusion, inflammatory mediators are also implicated, which can result in further neuronal injury [6, 7]. Accordingly, inflammation exists in all stages of acute ischemic stroke, from its inception through the progression and the final salvageable brain tissues repairing.

The recognition of inflammation in acute ischemic stroke spawned the application of inflammatory biomarkers to extend the investigation on stroke pathogenesis and prognosis as well as improvement on clinical therapeutics, especially by the concentration of peripheral inflammatory markers because of the conveniently operational availability and repeatability. Raised levels of markers of the acute inflammatory response after stroke are associated with poor outcomes [8]. Extensive studies demonstrated that the peripheral levels of white blood cell (WBC) [9–11], homocysteine (HCY) [12–15], and C-reactive protein [14, 16–18] strongly correlate with stroke severity and independently predict mortality and stroke recurrence in acute ischemic stroke patients. However, the effect of these biomarkers on intracranial stenosis is unsubstantial, despite significant attribution of

intracranial stenoocclusion to stroke severity. In addition, as a complicated process, inflammation usually involves multiple mediators, but the cooperative actions of these markers in acute ischemic stroke remained uncertain.

A better understanding of the significance of inflammatory mediators in intracranial stenosis and stroke severity in acute ischemic stroke would assist in the advanced therapeutic strategies. The aim of the present study was to clarify the contribution of inflammatory mediator level, including WBC, HCY, and high sensitivity C-reactive protein (hs-CRP), to categorized middle cerebral artery (MCA) stenosis as well as stroke severity by means of 1-year modified Rankin Scale (mRS).

2. Methods

2.1. Study Design and Participants. Institutional review board of Beijing Tiantan Hospital approved the study, and each participant provided an informed consent. From October 2007 to June 2009, we approached consecutive adult patients who presented with acute ischemic stroke or transient ischemic attack (TIA) with symptom onset within 7 days. We excluded the patients with atrial fibrillation or mRS > 2 before admission. We also excluded the patients who were clinically unstable or required close monitoring or were moribund, as well as physically or subjectively unable to comply with magnetic resonance examination or had severe comorbidity.

We recorded participants' demographics and risk factors (history of previous stroke, hypertension, diabetes mellitus (DM), hyperlipidemia, concurrent smoking, moderate-to-heavy drinking, and ischemic heart disease). Each patient underwent magnetic resonance imaging (MRI) and three-dimensional time of flight magnetic resonance angiography (3D TOF MRA) for the cerebral circulation. All patients underwent detailed clinical evaluation, for example, laboratory tests, National Institute of Health Stroke Scale (NIHSS) scores on admission or at discharge.

2.2. Imaging Evaluation. All patients underwent conventional MRI and MRA on a 3.0 T magnetic resonance scanner. Two stroke neurologists blind to subjects' clinical information reviewed the images. Disagreements of greater than 10% were further reviewed by a third reader who decided the final value.

Stenosis of MCA (M1/M2) was measured by WASID criteria [19] with Wiha DigiMax Digital Calipers 6′ (Germany) with a resolution of 0.01–0.03 mm for 0–100 mm and was classified as none or mild (<50%), moderate (50–69%), severe (70–99%), and occlusive (100%). If two or more stenoses were revealed, the stenotic severity of MCA would be identified by the most severe segment.

2.3. Follow-Up. All patients were assessed at 1 year after disease onset for clinical outcome by mRS (favorable (mRS = 1-2), poor (mRS = 3–6)) and recurrent stroke. Stroke recurrence was defined as functional deterioration in neurological status or a new sudden focal neurological deficit of vascular origin lasting more than 24 h, including recurrent ischemia or hemorrhage. Trained research personnel followed up patients over the telephone, using standard scripts to collect study data at the follow-up center.

2.4. Statistical Analysis. Continuous variables were summarized as mean ± SD or median (interquartile range, (IQR)). Categorical variables as gender and vascular risk factors were presented as n (%). Independent-samples t-test or Wilcoxon test was used for comparison of continuous variables. Comparison of categorical variables was analyzed by χ^2 test. In a multivariable analysis, stepwise logistic regression was used to evaluate the association of possible determinants and categorized MCA stenosis or 1-year mRS. Variables with a P value < 0.10 were included in the multivariate regression analysis.

All analyses were done with SAS software version 9.1 (SAS Institute Inc., Cary, NC, USA). For all tests, statistical significance was considered at the two-sided 5% level.

3. Results

From October 2007 to June 2009, a total of 1101 patients with acute ischemic stroke were admitted and 977 patients met the inclusion criteria. The clinical features of the patients are summarized in Table 1. The mean age was 59.97 ± 11.28 years, and 73.29% of the patients were men. The peripheral levels of inflammatory mediators were expressed by mean WBC ($6.91 \pm 1.96 \times 10^9$/L), HCY ($17.74 \pm 7.37$ μm mo/L), and median hs-CRP (2.6 mg/L, IQR (0.9–8.2)).

Table 2 presented patients' characteristics and inflammatory mediators by categorized MCA stenosis. In terms of peripheral level of inflammatory mediators, no significant differences were found between these four groups. The multivariate logistic regression analysis showed that none of these mediators predicted MCA stenoocclusion (not shown in table).

Of all the 977 patients, 952 completed 1-year follow-up and 25 cases (nearly 2.6%) were lost because of unable to contact. Patients with poor outcome had more MCA stenosis of ≥70% (13.71% versus 8.52%), more recurrent stroke (6.45% versus 2.27%), and higher levels of WBC, HCY, and hs-CRP. The variables with a P value < 0.10 were included in the stepwise multivariate regression analysis. In terms of inflammatory mediators, only hs-CRP was an independent predictive factor (OR 1.06, 95% CI 1.027–1.093, $P = 0.0003$). We performed multivariate analyses with hs-CRP (<1 mg/L, 1–3 mg/L and >3 mg/L), HCY (≤15 μmol/L and >15 μmol/L), and WBC (expressed by mean value, $\leq 6.91 \times 10^9$/L and $>6.91 \times 10^9$/L) as categorical variable (not shown in table) and got the same results as shown in Table 3.

We assessed the correlation of increased hs-CRP combined with elevated HCY or WBC with 1-year mRS. The patients were divided into three groups according to hs-CRP level (<1 mg/L, 1–3 mg/L and >3 mg/L). Furthermore, based on different combinations of peripheral HCY levels (≤15 μmol/L and >15 μmol/L) or WBC concentration (expressed by mean value, $\leq 6.91 \times 10^9$/L and $>6.91 \times 10^9$/L),

Table 1: Baseline characteristics of participants.

Demographics and characteristics	Overall (n = 977)
Age, years[#]	59.97 ± 11.28
Male	716 (73.29)
Duration between symptom onset and blood tests[&], day	3 (1–5)
Duration between symptom onset and MRI procedure[&], day	6 (4, 8)
Previous mRS score	
0	790 (80.86)
1	143 (14.64)
2	44 (4.5)
History of, yes (n, %)	
Previous cerebral ischemia, TIA, ICH, or SAH	266 (27.23)
Hypertension	788 (80.66)
Diabetes mellitus	409 (41.86)
Hyperlipidemia	802 (82.09)
Current smoking	484 (49.54)
Heavy-to-severe drinking	220 (22.52)
Ischemic heart disease	103 (10.54)
NIHSS score on admission[&]	4 (1, 8)
NIHSS score at discharge[&]	2 (0, 5)
MCA stenoocclusion	
None or <50%	615 (62.95)
50–69%	111 (11.36)
70%–99%	69 (7.06)
100%	182 (18.63)
Peripheral level of inflammatory mediators	
WBC[#], $\times 10^9$/L	6.91 ± 1.96
HCY[#], μmol/L	17.74 ± 7.37
hs-CRP[&], mg/L	2.60 (0.90–8.20)
Other laboratory findings on admission	
Hgb[#], g/L	142.01 ± 17.54
PLT[#], $\times 10^9$/L	213.03 ± 56.11
FBG[#], mmol/L	5.95 ± 2.21
Cr[#], μmol/L	78.50 ± 29.49
INR[#]	0.98 ± 0.14
HDL[#], mmol/L	1.12 ± 0.27
LDL[#], mmol/L	2.76 ± 0.84

[#]Continuous variables with normal distribution expressed as mean ± standard deviation.
[&]Continuous variables with nonnormal distribution expressed as interquartile range (IQR).
Other values were expressed as n (%).
SAH: subarachnoid hemorrhage; ICH: intracerebral hemorrhage; NIHSS: National Institute of Health Stroke Scale; WBC: white blood cell; Hgb: hemoglobin; PLT: platelet; FBG: free blood glycemia; Cr: creatinine; INR: international normalized ratio; HCY: homocysteine; hs-CRP: high sensitivity C-reactive protein; HDL: high-density lipoprotein; and LDL: low-density lipoprotein.

the patients were divided into 6 groups (Table 4). Adjusted by age, gender, history of DM and current smoking, NIHSS score on both admission and discharge, and the level of HDL, multivariate logistic regression model suggested a stronger correlation in poor outcome with combination of increased hs-CRP (>3 mg/L) and higher HCY (>15 μmol/L) (OR 4.487, 95% CI 1.994–10.098, $P = 0.0003$) or higher WBC (>6.91 $\times$ 10^9/L) (OR 3.174, 95% CI 1.713–5.884, $P = 0.0002$), compared to those combined with lower HCY (≤15 μmol/L) (OR 3.116, 95% CI 1.361–7.137, $P = 0.0072$) or lower WBC (≤6.91 $\times$ 10^9/L) (OR 2.381, 95% CI 1.284–4.415, $P = 0.0059$), respectively. We also found that, although the individual elevated level of WBC and HCY could not predict poor outcome, the combination of increased HCY (>15 μmol/L) and WBC (>6.91 $\times$ 10^9/L) dramatically independently predicts 1-year poor outcome (OR 1.879, 95% CI 1.158–3.05, $P = 0.0107$) (not shown in table).

4. Discussion

In this hospital-based, prospective, cohort study, we found three major contributions of inflammatory mediators to acute ischemic stroke. First, the peripheral levels of WBC, HCY and hs-CRP were comparable in patients with categorized MCA stenoocclusion. Second, patients with 1-year poor outcome had higher levels of WBC, HCY, and hs-CRP, but only hs-CRP is an independent predictor for unfavorable outcome. Third, the combination of any two of the increased hs-CRP, WBC, or HCY would have a stronger predictive value in poor outcome than individual elevated mediator.

Atherosclerosis is attributed to inflammation [1–4] and is commonly manifested as intracranial stenosis [20], so that it is necessary to investigate the potential interaction of inflammation with arterial stenosis. According to the first contribution described earlier, the inflammatory marker level did not parallel MCA stenotic severity which suggested a negative prognostic impact of inflammatory mediators on MCA stenosis. There may be several reasons underlying the negative correlation. Atherosclerosis, as a complex and systemic disease, may unequally induce intracranial stenosis [20], as well as the limitation of particular inflammatory mediator in describing atherosclerosis [2]. Moreover, history of previous stroke implied the usage of medicines for stroke prevention. We found that patients with MCA severe stenosis had the higher frequency accompanied with relative lower inflammatory mediator level, suggesting the possible effects of medicines for stroke prevention in biomarker level. Consistently, studies reported that medicines for stroke prevention may decrease inflammatory mediator, for example, aspirin [21, 22], clopidogrel [23], statins [24], folic acid [25], and vitamins B6 and B12 [25]. Accordingly, regardless of the fluctuated level of peripheral inflammatory markers, comprehensive information of interaction of inflammation with intracranial stenosis may be more critical by targeting patients with first-ever stroke.

By the second finding mentioned above, an increase of admission hs-CRP independently predicts 1-year poor outcome in acute ischemic stroke, which was consistent with prior studies [26, 27]. These results suggested the potential

Table 2: Patients' characteristics by categorized MCA stenoocclusion.

	MCA stenoocclusion				P value
	0–49% $n = 615$	50%–69% $n = 111$	70%–99% $n = 69$	100% $n = 182$	
Age, years[#]	59.97 ± 11.04	62.44 ± 11.67	63.01 ± 11.60	57.33 ± 11.22	0.0001
Male	438 (71.22)	81 (72.97)	51 (73.91)	146 (80.22)	0.1202
Duration between symptom onset and blood tests[&], day	3 (1–5)	3 (1–5)	3 (1–5)	3 (1–5)	0.7268
Duration between symptom onset and MRI procedure[&], day	6 (4, 8)	6 (4, 9)	7 (5, 8)	7 (4, 9)	0.0767
Previous mRS score					
0	506 (82.28)	89 (80.18)	48 (69.57)	147 (80.77)	0.1801
1	85 (13.82)	18 (16.22)	14 (20.29)	26 (14.29)	
2	24 (3.90)	4 (3.60)	7 (10.14)	9 (4.95)	
History of, yes (n, %)					
Previous cerebral ischemia, TIA, ICH, or SAH	152 (24.72)	29 (26.13)	28 (40.58)	57 (31.32)	0.0206
Hypertension	494 (80.33)	98 (88.29)	57 (82.61)	139 (76.37)	0.0899
Diabetes mellitus	253 (41.14)	58 (52.25)	34 (49.28)	64 (35.16)	0.0188
Hyperlipidemia	504 (81.95)	94 (84.68)	57 (82.61)	147 (80.77)	0.8626
Current smoking	308 (50.08)	50 (45.05)	24 (34.78)	102 (56.04)	0.0181
Heavy-to-severe drinking	136 (22.11)	28 (25.23)	15 (21.74)	41 (22.53)	0.9083
Ischemic heart disease	70 (11.38)	11 (9.91)	7 (10.14)	15 (8.24)	0.6731
NIHSS score on admission[&]	4 (1, 7)	4 (1, 7)	3 (1, 8)	7 (2, 11)	<0.0001
NIHSS score at discharge[&]	2 (0, 4)	2 (0, 4)	2 (1, 5)	4 (1, 8)	<0.0001
Peripheral level of inflammatory mediators					
WBC[#], $\times 10^9$/L	6.89 ± 1.90	6.80 ± 1.94	6.50 ± 1.75	7.20 ± 2.20	0.0583
HCY[#], μmol/L	17.52 ± 7.29	17.56 ± 6.58	17.21 ± 6.57	18.78 ± 8.31	0.2325
hs-CRP[&], mg/L	2.4 (0.9, 6.7)	2.75 (0.8, 9.5)	2.1 (0.7, 7.2)	3.8 (1.1, 0.2)	0.0580
Other laboratory findings on admission					
Hgb[#], g/L	141.84 ± 18.21	141.24 ± 18.47	139.25 ± 14.08	144.10 ± 15.67	0.2094
PLT[#], $\times 10^9$/L	214.67 ± 56.11	212.95 ± 60.36	202.15 ± 41.93	211.70 ± 57.98	0.3657
FBG[#], mmol/L	5.92 ± 2.27	6.21 ± 2.32	6.26 ± 2.16	5.78 ± 1.94	0.2566
Cr[#], μmol/L	78.27 ± 33.06	79.83 ± 22.80	76.84 ± 23.25	79.06 ± 21.62	0.9094
INR[#]	0.98 ± 0.08	1.00 ± 0.35	0.97 ± 0.05	0.97 ± 0.05	0.3831
HDL[#], mmol/L	1.15 ± 0.28	1.11 ± 0.31	1.05 ± 0.23	1.08 ± 0.25	0.0038
LDL[#], mmol/L	2.76 ± 0.80	2.75 ± 0.83	2.77 ± 0.95	2.75 ± 0.92	0.9987
Recurrent stroke, yes	17 (2.76)	6 (5.41)	5 (7.25)	6 (3.30)	0.1649
one-year mRS					
0–2	471 (79.16)	78 (70.27)	44 (63.77)	111 (62.71)	<0.0001
3–6	124 (20.84)	33 (29.73)	25 (36.23)	66 (37.29)	

[#]Continuous variables with normal distribution expressed as mean ± standard deviation.
[&]Continuous variables with nonnormal distribution expressed as interquartile range (IQR).
Other values were expressed as n (%).
SAH: subarachnoid hemorrhage; ICH: intracerebral hemorrhage; NIHSS: National Institute of Health Stroke Scale; WBC: white blood cell; Hgb: hemoglobin; PLT: platelet; FBG: free blood glycemia; Cr: creatinine; INR: international normalized ratio; HCY: homocysteine; hs-CRP: high sensitivity C-reactive protein; HDL: high-density lipoprotein; and LDL: low-density lipoprotein.

benefit of neuroprotective therapeutics by anti-inflammation in acute ischemic stroke. Unfortunately, secondary prevention of cardiovascular disease by neuroprotection against adverse clinical outcomes was still uncertain [25, 28, 29]. Interestingly, we found the predictive value of increased PLT level in 1-year poor outcome of acute ischemic stroke. As known to us, circulating platelet mass (PLT count × mean platelet volume (MPV)) is normally kept constant [30], and prior reports indicated predictive value of high MPV in ischemic stroke [31, 32]. These pieces of information implied reasonable possibility of decrease instead of increase of PLT level in patients with poor outcome. For the inconsistency,

Table 3: Univariate and multivariate analysis in patients' 1-year outcome.

	one-year mRS		P value	OR (95% CI)	Multivariate P value
	0–2 (favorable), $N = 704$	3–6 (poor), $N = 248$			
Age, years[#]	59.16 ± 11.02	62.23 ± 11.57	0.0002	1.022 (1.006–1.039)	0.0080
Male	526 (74.72)	173 (69.76)	0.1285		
Duration between symptom onset to blood tests[&], day	3 (1–5)	3 (1–5)	0.1990		
Duration between symptom onset to MRI procedure[&], day	6 (4, 8)	6 (4, 8)	0.1969		
Previous mRS score					
0	588 (83.52)	178 (71.77)	0.0002	—	—
1	92 (13.07)	51 (20.56)			
2	24 (3.41)	19 (7.66)			
History of, yes (n, %)					
Previous cerebral ischemia, TIA, ICH, or SAH	181 (25.71)	81 (32.66)	0.0351	—	—
Hypertension	561 (79.69)	207 (83.47)	0.1780		
Diabetes mellitus	303 (43.04)	99 (39.92)	0.3922		
Hyperlipidemia	589 (83.66)	193 (77.82)	0.0389	—	—
Current smoking	355 (50.43)	116 (46.77)	0.3226		
Heavy-to-severe drinking	155 (22.02)	57 (22.98)	0.7530		
Ischemic heart disease	81 (11.51)	20 (8.06)	0.1302		
NIHSS score on admission[&]	3 (1, 6)	7 (3, 11)	<0.0001	1.063 (1.003–1.127)	0.0397
NIHSS score at discharge[&]	2 (0, 3)	5 (2, 9)	<0.0001	1.123 (1.045–1.208)	0.0017
MCA stenoocclusion					
None or <50%	471 (66.90)	124 (50)	<0.0001	—	—
50–69%	173 (24.57)	90 (36.29)		1.708 (1.028–2.840)	0.0389
70%–99%	49 (6.96)	31 (12.50)		2.073 (1.107–3.882)	0.0228
100%	11 (1.56)	3 (1.21)		1.660 (1.066–2.585)	0.0249
Peripheral level of inflammatory mediators					
WBC[#], $\times 10^9$/L	6.74 ± 1.87	7.38 ± 2.15	<0.0001	—	—
HCY[#], μmol/L	17.45 ± 7.29	18.59 ± 7.39	0.0398	—	—
hs-CRP[&], mg/L	2.0 (0.8, 5.75)	6.45 (1.9, 12.3)	<0.0001	1.060 (1.027–1.093)	0.0003
Other laboratory findings on admission					
Hgb[#], g/L	142.58 ± 17.36	140.69 ± 17.69	0.1450		
PLT[#], $\times 10^9$/L	210.62 ± 55.36	218.53 ± 57.83	0.0577	1.004 (1.001–1.007)	0.0100
FBG[#], mmol/L	5.91 ± 2.25	6.10 ± 2.13	0.2457		
Cr[#], μmol/L	79.43 ± 31.67	76.25 ± 23.18	0.0960		
INR[#]	0.97 ± 0.07	0.99 ± 0.24	0.1911		
HDL[#], mmol/L	1.13 ± 0.28	1.11 ± 0.28	0.4684		
LDL[#], mmol/L	2.76 ± 0.85	2.77 ± 0.78	0.9366		

[#]Continuous variables with normal distribution expressed as mean ± standard deviation.
[&]Continuous variables with nonnormal distribution expressed as interquartile range (IQR).
Other values were expressed as n (%).
SAH: subarachnoid hemorrhage; ICH: intracerebral hemorrhage; NIHSS: National Institute of Health Stroke Scale; WBC: white blood cell; Hgb: hemoglobin; PLT: platelet; FBG: free blood glycemia; Cr: creatinine; INR: international normalized ratio; HCY: homocysteine; hs-CRP: high sensitivity C-reactive protein; HDL: high-density lipoprotein; and LDL: low-density lipoprotein.

Table 4: Multivariate analysis of 1-year outcome in hs-CRP combined with HCY or WBC.

hs-CRP (mg/L)	HCY (μmol/L)	OR (95% CI)	P value	WBC ($\times 10^9$/L)	OR (95% CI)	P value
<1	≤15	—	—	≤6.91	—	—
	>15	1.973 (0.781–4.986)	0.1506	>6.91	1.685 (0.714–3.976)	0.2336
1–3	≤15	1.860 (0.758–4.564)	0.1753	≤6.91	1.618 (0.833–3.146)	0.1557
	>15	2.295 (0.944–5.582)	0.0670	>6.91	1.042 (3.146–2.288)	0.9182
>3	≤15	3.116 (1.361–7.137)	0.0072	≤6.91	2.381 (1.284–4.415)	0.0059
	>15	4.487 (1.994–10.098)	0.0003	>6.91	3.174 (1.713–5.884)	0.0002

Adjusted by age, gender, NIHSS score on admission/discharge, history of DM and current smoking, and the level of HDL.
WBC level was expressed by mean value as shown in Table 1.

further information by dynamic monitoring of PLT level in ischemic stroke is necessary.

In recent years, inflammatory mediators have been individually investigated intensively in patients with ischemic stroke. However, there has been little attention given to the cooperative role of these markers. Based on the third contribution, we observed the cooperative impact of increased WBC, HCY, and hs-CRP on clinical outcome by stronger association of any two increased mediators, instead of individual elevated mediator, with 1-year poor outcome. One possible reason was that, in the complex process of inflammation, multiple mediators may be dependent on inflammation-related mechanisms in the course of acute cerebral ischemia, which was described in a prior study by small sample size [33]. In the treatment strategy of cerebrovascular disease, whether lowering the mediator level reduces the risk of cardiovascular events was controversial [25, 28]. Based on the observation of the superimposed effect of inflammatory markers, we suspected that detection and intervention of multiple inflammatory markers might have greater significance than single one in stroke mechanism and treatment formulation in neuroprotection. However, there was no standard for the prespecified targets in inflammatory mediators according to current guidelines in stroke prevention, which suggested that further study should be conducted for detailed information on cooperative inflammatory impact on ischemic stroke.

We had a few limitations in this study. First, we used 3D TOF MRA to evaluate MCA stenosis. Although MRA is not the gold standard for assessing intracranial stenosis, hierarchical evaluation instead of detailed value of stenotic severity improved the measuring accuracy to some extent. Second, functional outcome might be associated with not only MCA but also with other intracranial large arteries stenosis, which possibly generated an analysis bias. Third, because of the fluctuant levels of inflammatory markers, one-time examination of plasma level might confound the mediator concentration. Finally, medicines given to patients for stroke prevention might affect inflammatory mediator level and disturb the analysis of the inflammatory impact on MCA stenosis.

5. Conclusion

The prognostic value of increased hs-CRP, especially the combination of increased inflammatory markers in predicting 1-year poor outcome in acute ischemic stroke, might provide insight information into stroke mechanism and treatment strategy, particularly in neuroprotection, for acute ischemic stroke.

Authors' Contribution

Xiping Gong and Xinying Zou contributed equally to this paper.

Conflict of Interests

The authors declared that there was no conflict of interests relevant to what they wrote.

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Review Article

Regulation of Immune Cell Infiltration into the CNS by Regional Neural Inputs Explained by the Gate Theory

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The central nervous system (CNS) is an immune-privileged environment protected by the blood-brain barrier (BBB), which consists of specific endothelial cells that are brought together by tight junctions and tight liner sheets formed by pericytes and astrocytic end-feet. Despite the BBB, various immune and tumor cells can infiltrate the CNS parenchyma, as seen in several autoimmune diseases like multiple sclerosis (MS), cancer metastasis, and virus infections. Aside from a mechanical disruption of the BBB like trauma, how and where these cells enter and accumulate in the CNS from the blood is a matter of debate. Recently, using experimental autoimmune encephalomyelitis (EAE), an animal model of MS, we found a "gateway" at the fifth lumber cord where pathogenic autoreactive CD4+ T cells can cross the BBB. Interestingly, this gateway is regulated by regional neural stimulations that can be mechanistically explained by the gate theory. In this review, we also discuss this theory and its potential for treating human diseases.

1. Mechanism for BBB Breakdown in Autoimmunity of the CNS

The blood-brain barrier (BBB) in blood vessels is known to strictly limit the inflow of substances like proteins and cells from the bloodstream into the CNS (Figure 1), thereby maintaining a homeostatic environment for surrounding neurons and glia cells, a property different from that in peripheral organs. The BBB is formed and maintained by endothelial cells and corresponding tight junctions formed by claudins and occludins in collaboration with pericytes, microglial cells, macrophages, and astrocytes [1, 2]. BBB dysfunction is known to be associated with chronic neurodegenerative disorders, such as Parkinson's disease and Alzheimer's disease, and autoimmune diseases in the CNS [3, 4]. An increasing number of studies have shown that one cause of a dysfunctional BBB is inflammatory cytokines. For example, tumor-necrosis factor α (TNFα), interleukin-(IL-)1β, and IL-17A have all been reported to loosen the BBB [5]. In particular, IL-17A is known to disrupt the BBB *in vitro* and *in vivo*. Huppert et al. reported that IL-17A-induced BBB dysfunction involves the formation of reactive oxygen species by NADPH oxidase and xanthine oxidase and that these species lead to the down regulation of tight junction molecules and the activation of the endothelial contractile machinery *in vitro* [6]. In addition, Kebir et al. reported that treatment with IL-17A increases the protein permeability of human brain endothelial cells and that this permeability is associated with a decrease in the expression of occludin and ZO-1 [7]. A role of IL-17A in BBB disruption has also been found using experimental autoimmune encephalomyelitis (EAE) mice, an animal model of multiple sclerosis (MS), *in vivo*. In this model, the major source of IL-17A is type-17 helper T (Th17) cells, particularly autoreactive ones. EAE is significantly suppressed in IL-17A-deficient mice. Instead, these mice exhibit delayed onset, reduced maximum severity

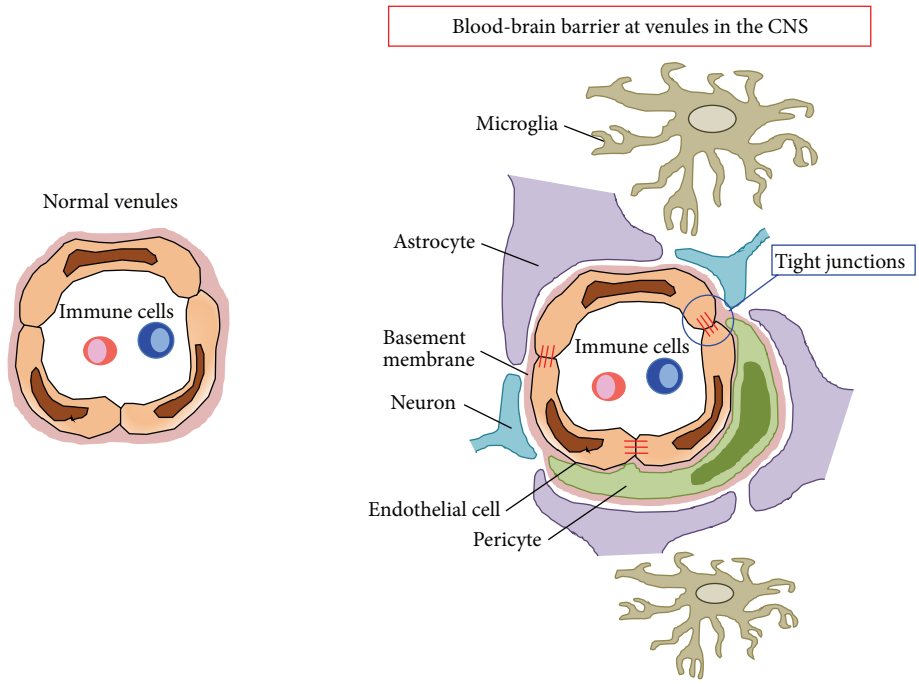

Figure 1: Venules at the blood-brain barrier in the CNS and other organs. Venules at the blood-brain barrier consist of specific endothelial cells that are brought together by tight junctions and tight liner sheets formed by pericytes and astrocytic end-feet. These are not present in normal venules (left).

scores, ameliorated histological changes, and early recovery [8]. Additionally, an adoptive transfer model in which helper T cells obtained from myelin oligodendrocyte glycoprotein (MOG) immunized mice were infused into naïve recipients indicated that IL-17A derived from CD4+ T cells is critical for the induction of EAE [8]. In addition, MOG-reactive Th17 cells obtained from MOG-immunized IL-17A-deficient mice were unable to infiltrate the lumber level of spinal cord in the same model (see the following for details) [9]. Furthermore, it was shown that the adoptive transfer of Th17 cells from ovalbumin-specific T cell receptor transgenic mice, which are unable to recognize CNS antigens, does not pass the BBB and migrate into CNS, whereas cotransfer of these Th17 cells with MOG-reactive Th17 cells leads to the accumulation of both types of Th17 cells in the CNS (our unpublished data and [10]), which strongly suggests that antigen recognition of Th17 cells is required for severe disruption of the BBB. Although antigen presentation inside the CNS has suggested through observation that the infusion of ovalbumin peptide-loaded antigen-presenting cells into cerebrospinal fluids induces an accumulation of ovalbumin-specific Th17 cells in the CNS [10], the type of antigen-presenting cells and location where antigen presentation takes place under physiological conditions remain elusive. Nevertheless, these results suggest that IL-17A expressing Th17 cells, which recognize CNS antigens, have a major impact on breaching the BBB, in part by decreasing tight junction molecules.

2. Neuroimmune Interactions Responsible for Inflammation in the CNS

In the previous section, we discussed the relationship between inflammatory cytokines such as IL-17A and disruption of the BBB. This section focuses on the gateway for which pathogenic CD4+ T cells enter the CNS. In patients with MS, common early symptoms include vision problems and tingling, followed by many neurological signs as the disease progresses. It is known that inflammation sites in MS are found in specific regions of white matter, including the brainstem, the optic nerve, the cerebellum, the long motor, and sensory tracts of the spinal cord [11]. This fact suggests that some CNS regions might be more vulnerable to autoimmune attacks. One hypothesis proposes that chemokine recruitment of pathogenic autoreactive T cells is more abundant in these regions. Among the many chemokines, *CCL20* is of particular interest, as it attracts Th17 cells that express CCR6, a receptor for CCL20. Reboldi et al. reported that mice lacking CCR6 are highly resistant to EAE and that the choroid plexus, a specialized epithelial structure in the brain known to produce cerebrospinal fluids, expresses CCL20 constitutively, an effect that acts as an attractant for the first wave of CCR6+ Th17 cells [12]. In that same study, however, EAE was induced using the complete Freund's adjuvant, which is widely used to generate active immunization in animals but is also an inducer of systemic inflammation and has many side effects including fever, motor neuron dysfunction such

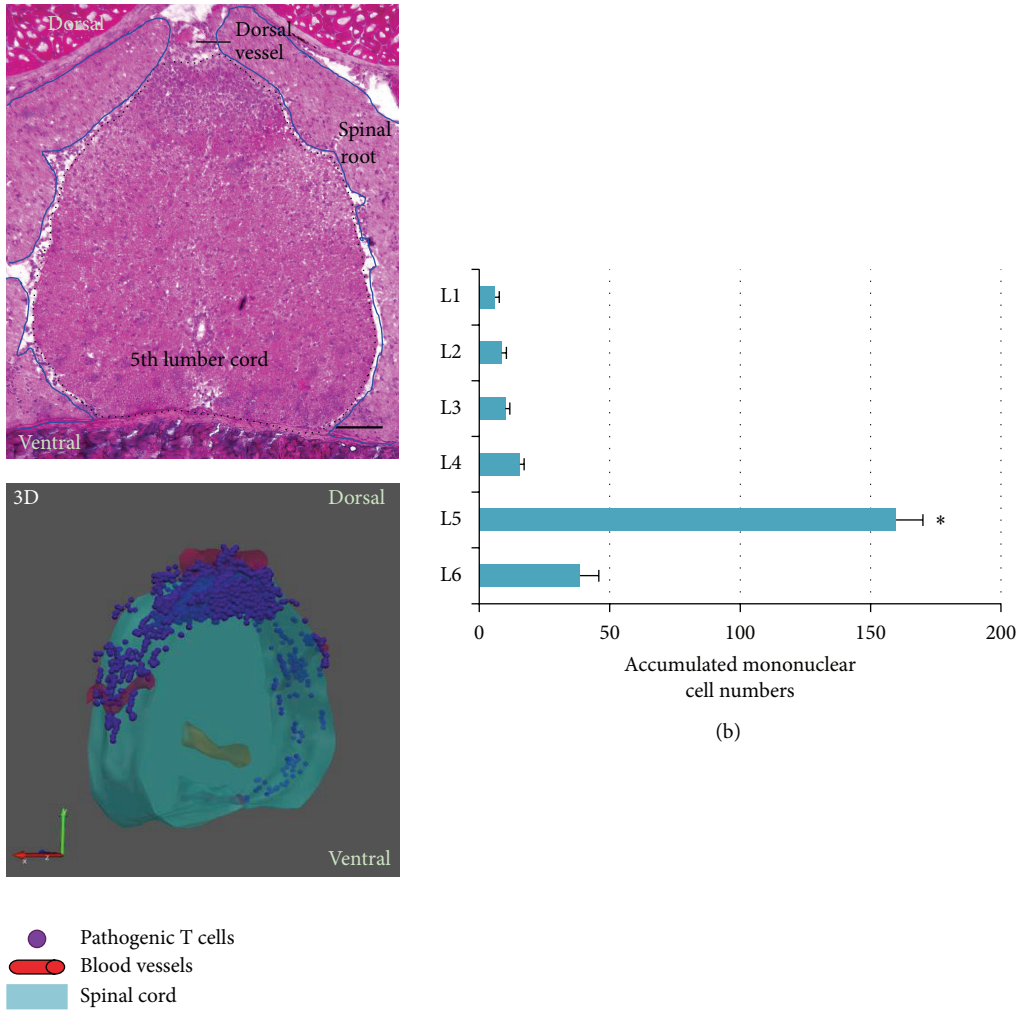

Figure 2: The fifth lumber cord is a gateway into the CNS. A cross section of the fifth lumber (L5) cord (a) and actual cell numbers of mononuclear cells accumulated in each lumber cord segment (b) at a preclinical phase of EAE (5 days after pathogenic Th17 transfer). A 3D picture based on ten serial sections of L5 is also shown.

as paralysis, and apoptosis. These side effects could affect the pathophysiological status of the brain and spinal cords resulting in different conclusions from the steady state.

We recently found a "gate" past the BBB in the spinal cord that autoreactive Th17 cells in the bloodstream can exploit to enter the CNS. To make this discovery, we first utilized an adoptive transfer model to induce EAE in which Th17 cells obtained from MOG-immunized mice were infused into naïve recipient mice to maintain CNS quiescence. In this adoptive transfer model, we found that MOG-reactive Th17 cells preferentially accumulated in the fifth lumber (L5) cord rather than the brain or other levels of the spinal cords at the earliest phase of EAE (day 5 after T cell transfer) [9] (Figure 2). This finding fits well with a typical clinical EAE sign in which the tail is first affected. We also found that blood vessel tracks in L5 are altered due to the formation of edema in the L5 cord by using a supersensitive MRI (data not shown). Consistent with these results, *Ccl20* mRNA levels were highest in the dorsal venules of L5 compared with those from other spinal cords, and the transfer of CCR6-deficient Th17 cells did not accumulate in the L5 region. Interestingly, even in naïve animals without Th17 transfer, mRNA levels of *Ccl20* and many other chemokines were specifically upregulated in the dorsal venules of L5. Therefore, dorsal venules in the L5 spinal cord have special properties in diseased as well as healthy conditions.

We previously found a mechanism for the hyperinduction of inflammatory chemokines and cytokines in nonimmune cells such as fibroblasts, endothelial cells, and epithelial cells using a rheumatoid arthritis model. The mechanism is driven by a simultaneous activation of two transcription factors, NF-κB and STAT3. Thus, it was named the "inflammation amplifier", because hyperactivation of NF-κB by activated STAT3 induces large amounts of NF-κB-targeted chemokines and chemotactic factors to promote the recruitment of immune cells (Figure 3). *Ccl20* is one such target chemokine and is

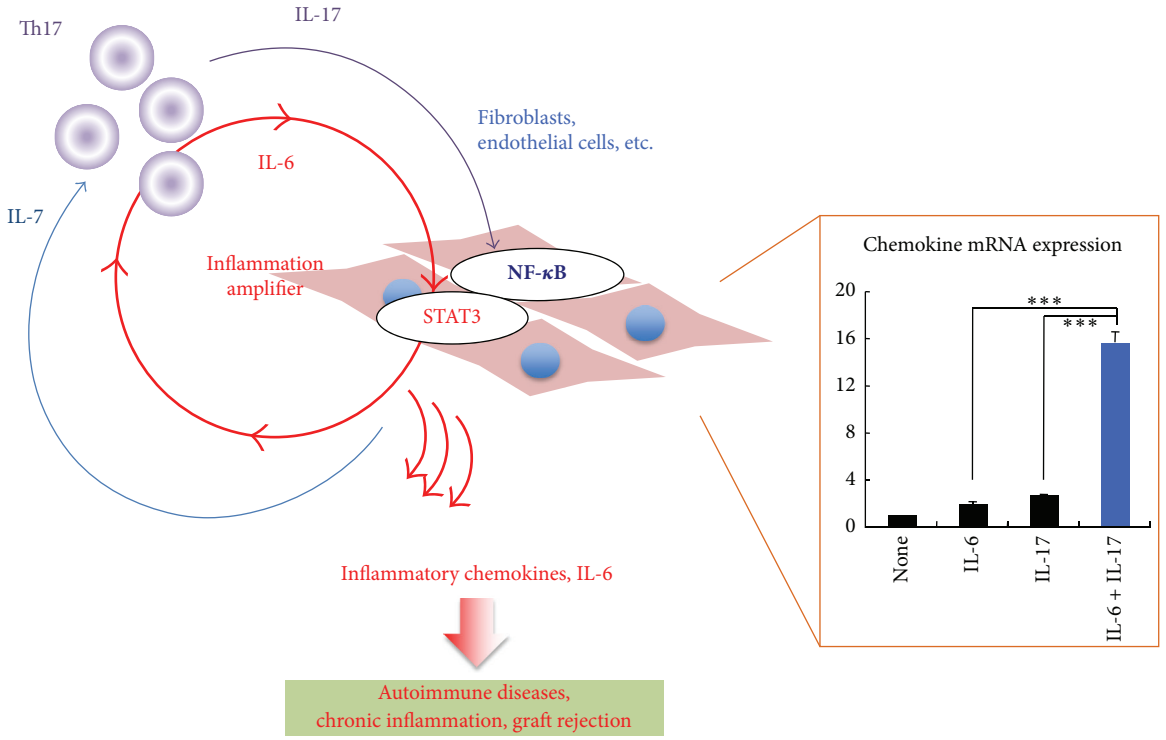

Figure 3: The inflammation amplifier is defined as a mechanism that triggers the hyperinduction of cytokines and chemokines in nonimmune cells and leads to many diseases. This induction is induced by the simultaneous activation of NF-κB and STAT3. IL-7 from nonimmune cells also contributes to enhancing the inflammation amplifier by generating Th17 cells and/or sustaining their survival. The inflammation amplifier is essential for the pathogenesis of F759 arthritis, EAE, and chronic graft rejection. The bar graph on the right indicates typical expression statuses of chemokines and IL-6 after inflammation amplifier activation (see the blue bar).

found in vascular endothelial cells. Given that chemokine expressions are elevated in L5 dorsal venules, we hypothesized a role for the inflammation amplifier. The activation status of NF-κB and STAT3 is indeed higher in L5 dorsal venules than other lumber cords even in naïve healthy mice. The elevated *Ccl20* mRNA levels at L5 vessels were decreased in mice devoid of the inflammation amplifier such as IL-6-deficient mice and endothelial cell-specific IL-6 receptor (gp130) deficient mice. Even under healthy conditions, it is known that some immune cells are present in the CNS, suggesting that there may be a gate to enter the restricted tissue regardless. In this respect, it is tempting to speculate that low-grade activation of the inflammation amplifier at L5 dorsal venules creates the gate by inducing certain levels of chemokines, although further studies are required for direct evidence that links activation of the inflammation amplifier with immunity homeostasis in the CNS.

Upon discovering the L5 cord as the entry site of autoreactive Th17 cells at the initial phase of EAE [9], we searched for reasons that would make L5 ideal for this gate. The answer came from physiological responses to gravitation stimuli. Soleus muscles are constantly stimulated by gravitational forces, and the dorsal root ganglia of their sensory neurons are located beside the L5 cord [13]. We hypothesized that frequent stimulation of the soleus muscles by gravity could induce activation of the inflammation amplifier via sensory nerves. In experiments that had healthy normal mice suspended from their tails so that only the forelimbs could touch the ground and the hind legs were released from gravitational forces, MOG-reactive Th17 cells no longer accumulated at L5 (Figure 4). Instead, they accumulated at cervical cords, indicating that burdening the arm muscles with body weight opened a new gateway for immune cells [9]. Consistent with this observation, tail suspension significantly inhibited *Ccl20* mRNA expression in L5 dorsal blood vessels and decreased the expression of the neural activation marker, c-Fos, in L5 dorsal root ganglia. In addition, when the soleus muscles of tail-suspended mice were artificially stimulated by weak electric pulses, *Ccl20* expression, MOG-specific Th17 accumulation, and c-Fos levels were restored at L5 (Figure 4(b) and not shown). These data strongly suggest that neural activation by an antigravitational response plays a role in the activation of the inflammation amplifier, leading to the expression of many chemokines including Th17-attracting *Ccl20* in L5 dorsal blood venules [9].

What mechanisms do afferent sensory neurons from the soleus muscle use to regulate the status of blood venules at L5? Although a precise neural network remains unclear, we have shown sympathetic nerves to be involved. Blood flow speed at L5 dorsal venules became slower when mice are tail suspended, while electronic stimulation of the soleus muscles increases the flow, suggesting that automatic nerves including sympathetic ones are involved in the response. On the other hand, blood flow speeds in blood vessels other than

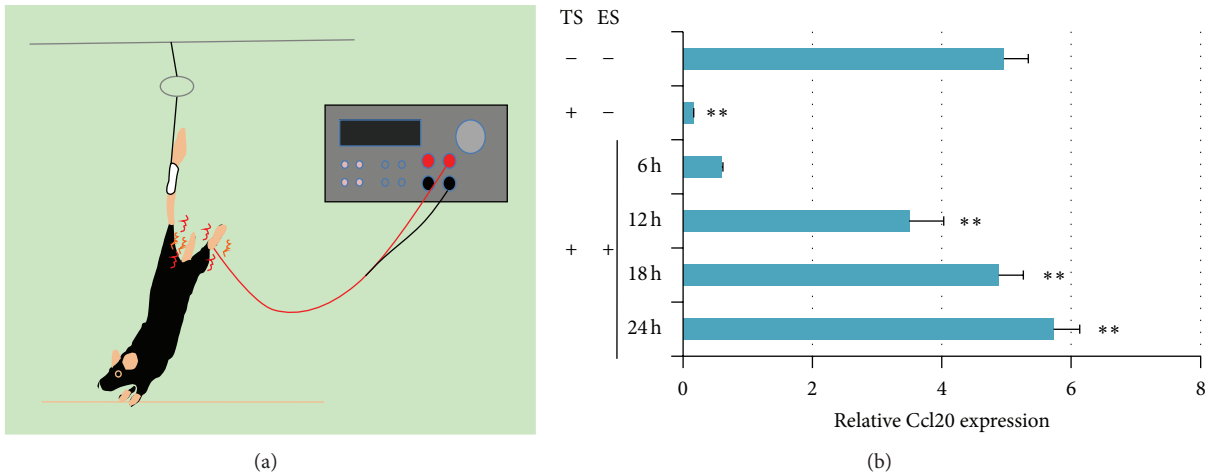

Figure 4: Neural stimulation-mediated activation of the inflammation amplifier creates a gateway into the CNS via chemokine production. Schematic illustration of the tail suspension model (a). A string is fastened to the roof of the cage at a height that allows the forelimbs to support body weight but prevents the hindlimbs from touching any part of the cage. Release from gravitational stimuli caused by tail suspension (TS) results in a decrease of Ccl20 levels at the L5 dorsal venules. Electric stimulation (ES) during TS restores the levels in a time-dependent manner (b).

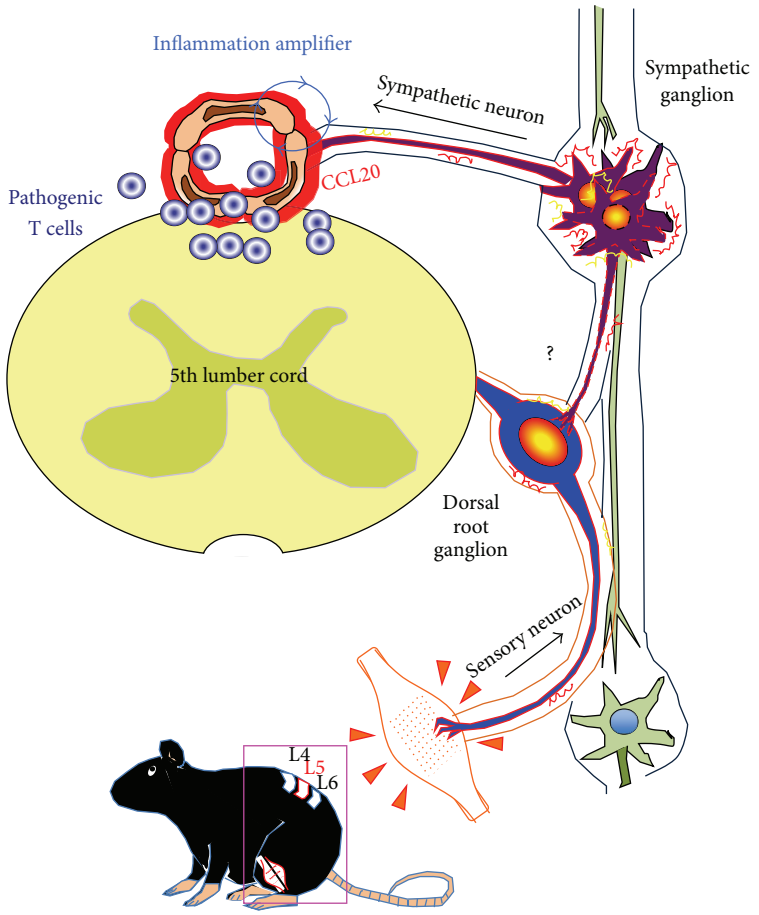

Figure 5: Schematic representation of neural stimulation-mediated activation of the inflammatory amplifier. Neural signals from the soleus muscles caused by gravitational stimulation reache the L5 dorsal root ganglion. Subsequent activation of sympathetic nerves alters the status of endothelial cells in L5 dorsal venules to enhance the inflammation amplifier, which leads to the production of chemokines including CCL20. Norepinephrine acts as a mediator between the neural signal and activation of the inflammation amplifier. The neural network determining communication between soleus muscle-derived sensory neurons and sympathetic neurons that reach L5 is not currently defined (depicted with a question mark).

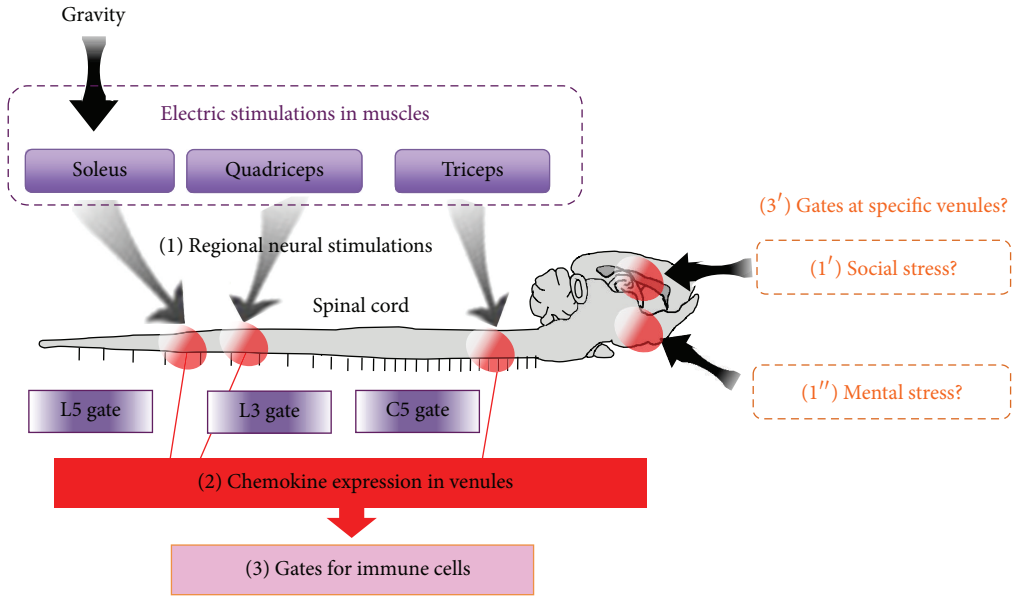

Figure 6: Each neural stimulation establishes a regional gate of immune cells in the CNS. Gravity or electric stimulations to soleus muscles can open the L5 gateway via regional neural stimulations-mediated chemokine expression, electronic stimulation of the quadriceps followed by neural stimulation establishes the L3 gateway, and gateways in the fifth cervical to fifth thoracic cord vessels were created by electric stimulations to triceps brachii. Therefore, it is reasonable that neural stimulations induced by stresses like social and mental ones might establish gates in some specific sites of the brain.

the L5 region, such as femoral vessels, brain surface vessels, and the portal vein, are not affected by tail suspension. Furthermore, treatment with atenolol, a β1 adrenergic receptor antagonist, or prazosin, an α1 adrenergic receptor antagonist, significantly suppresses *Ccl20* mRNA expression, NF-κB activation, and MOG-reactive Th17 accumulation at L5 vessels and also suppresses clinical signs of EAE [9]. Consistent with these *in vivo* results, the addition of norepinephrine to a culture of endothelial cell lines enhances the inflammation amplifier based on IL-6 and Ccl20 expressions. Thus, antigravity responses of the soleus muscles result in sympathetic nerve stimulation, which creates a gateway of immune cells to pass though the CNS via L5 dorsal venules [9]. Based on these findings, we propose that MOG-reactive, disease-causing Th17 cells make use of the L5 gateway to infiltrate the CNS and induce local inflammation by producing cytokines like IL-17A, which further induces chemokines through the inflammation amplifier and results in chronic inflammation of the CNS (Figure 5). At a later phase of EAE, transferred Th17 cells are also found in the brain including regions like the choroid plexus and cerebellum. Whether these Th17 cells move within the cerebrospinal fluid to finally reach the brain and/or enter the brain directly from the circulation is unclear. However, in the brain of EAE mice with advanced clinical scores, transferred Th17 cells are not uniformly localized. Rather, they tend to accumulate in specific areas of the brain (our unpublished data), which argues for the involvement of neural activation and subsequent breach of the BBB in these areas. Using this logic, we speculate that the relatively high incidence of vision dysfunction during the initial phases of MS in humans might be due to the persistent visual stimulation in our everyday lives that activates the optical nerves and unlocks the gate nearby.

Other neuroimmune interactions have been reported by other groups. The Kevin Tracey group, which is a pioneer in this field, has demonstrated that vagus nerve stimulation suppresses the release of proinflammatory cytokines through the nicotinic acetylcholine receptor α7 subunit and identified a subset of T cells producing acetylcholine that can relay neural signals [14–16]. Acetylcholine is also produced by other immune cells including B cells, which have an impact on innate immunity [17]. Cao et al. reported that mice reared in a larger cage with toys and more mice, that is, an enriched environment, are resistant to tumor burden in a manner dependent on sympathetic nerve activation via the BDNF/leptin axis [18]. Nguyen et al. reported a relationship between catecholamines, alternative macrophages, and thermogenesis, finding that exposure to cold temperatures rapidly promotes alternative activation of adipose tissue macrophages, leading to a secretion of catecholamines that induces thermogenic gene expressions in brown adipose tissue and lipolysis in white adipose tissues [19]. Additionally, Hassan et al. showed that behavioral stress promotes prostate cancer development by inhibiting the apoptosis of tumor cells via the β2-adrenergic receptor [20]. Therefore, a strategy to modulate neuroimmune interactions may prove a promising approach for therapeutic interventions against many inflammatory diseases.

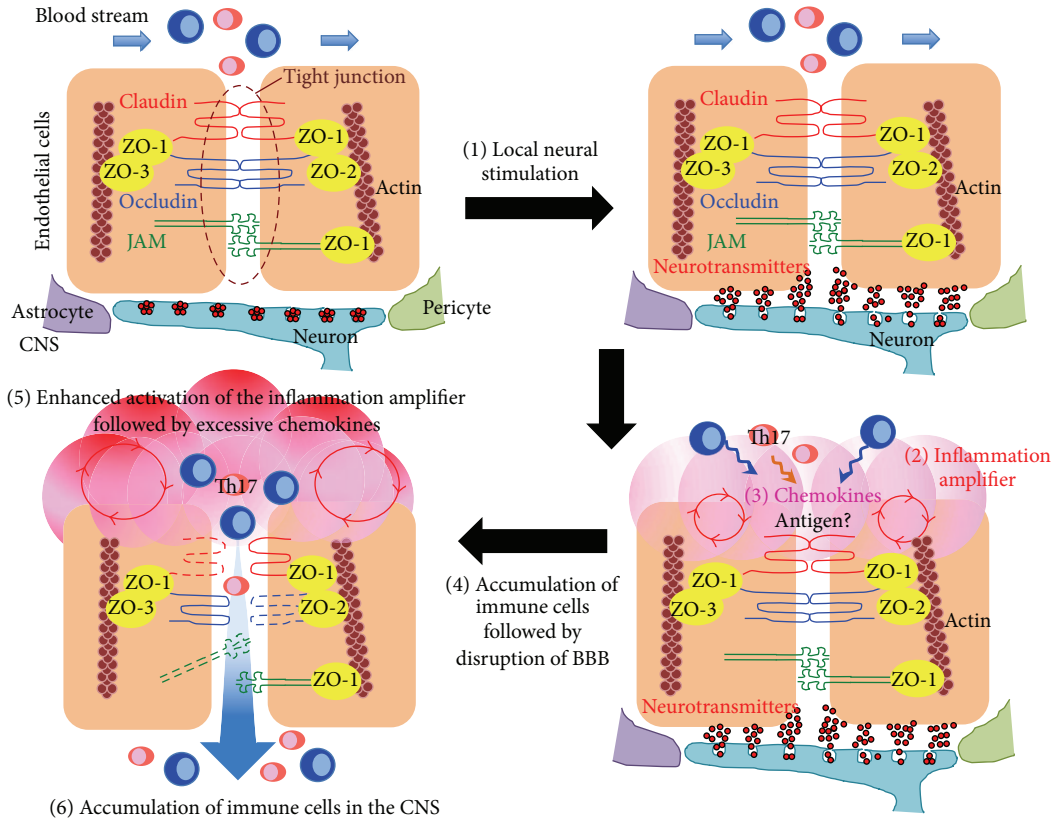

FIGURE 7: Schematic representation of the gate theory in the CNS. The gate theory describes how regional neural stimulations direct immune cell infiltration into target organs by crossing gates located at various blood vessels. Immune cells in the blood stream cannot enter the target organs due to homeostasis of the venules (upper left). Regional neural stimulation secretes neurotransmitters including norepinephrine into the endothelial cells of the venules (upper right). This stimulation activates the inflammation amplifier in the endothelial cells followed by increasing local chemokine expression (bottom right). The accumulation of immune cells disrupts the BBB, which allows immune cells to enter the CNS (bottom left).

3. The Gate Theory

The dorsal venules of the L5 spinal cord have been found to act as a gateway for MOG-reactive Th17 cells to accumulate into the CNS in an adoptive transfer model of murine EAE during steady state. Gravity or electric stimulations to soleus muscles can open the L5 gateway, as described above. We extended these findings by applying electric pulses to other muscle regions. Electronic stimulation of the quadriceps or thigh muscles, which are known to be regulated by L3 dorsal-root ganglion neurons, increased the expression of *Ccl20* mRNA in L3 cord vessels in mice. Similarly, chemokine levels in the fifth cervical to fifth thoracic cord vessels were upregulated by electric stimulations to epitrochlearis/triceps brachii (upper arm muscles), which are controlled by neurons located at the corresponding spinal regions (Figure 6) [9]. Based on these findings, we proposed "the gate theory", which describes how regional neural stimulations direct immune cell infiltration into target organs by crossing gates located at various blood venules (Figure 7). Investigations on whether the gate theory can be applied to tissues other than the CNS are ongoing. The ability to manipulate these gates at targeted regions in the body is expected to have significant clinical benefits, as closing them should ameliorate autoimmune inflammation in the target organ without any systemic immune suppression, while opening these gates near surrounding tumors may enhance cancer immunotherapy effects. With such medical promise, much effort is needed to identify the precise molecular mechanisms for gating.

Conflict of Interests

The authors declare that they have no conflicting financial interests.

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Review Article

Are Onconeural Antibodies a Clinical Phenomenology in Paraneoplastic Limbic Encephalitis?

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Paraneoplastic neurological syndromes (PNSs) occur in patients with cancer and can cause clinical symptoms and signs of dysfunction of the nervous system that are not due to a local effect of the tumor or its metastases. Most of these clinical syndromes in adults are associated with lung cancer, especially small cell lung cancer (SCLC), lymphoma, and gynecological tumors. The finding of highly specific antibodies directed against onconeural antigens has revolutionized the diagnosis and promoted the understanding of these syndromes and led to the current hypothesis of an autoimmune pathophysiology. Accumulating data strongly suggested direct pathogenicity of these antibodies. The field of PNS has expanded rapidly in the past few years with the discovery of limbic encephalitis associated with glutamic acid decarboxylase (GAD) 65, the voltage (VGKC-gated potassium channel) complex, the methyl (N-NMDA-D-aspartate), alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and gamma aminobutyric acid (GABA) (B) receptors, and so forth. Despite this, the clinical spectrum of these diseases has not yet been fully investigated. The clinical importance of these conditions lies in their frequent response to immunotherapies and, less commonly, their association with distinctive tumors. This review provides an overview on the pathogenesis and diagnosis of PNS, with emphasis on the role of antibodies in limbic encephalitis.

1. An Overview of Paraneoplastic Neurological Syndromes

The idea that neural cells can be the target of autoimmune responses mediated by antibodies is still not well recognized in the medical community [1]. Paraneoplastic neurological syndromes (PNSs) are rare dysfunctions of the nervous system in patients with cancer, which are not due to a local effect of the tumor or its metastases. Most of these clinically defined syndromes in adults are associated with lung cancer, especially small cell lung cancer (SCLC), lymphoma, or gynecological tumors. Antibodies directed against onconeural antigens are frequently detected in patients with PNS. So far, these antibodies have been thought to be the only markers of the disease and not to play a role in the pathophysiology. However, the recent description of antibodies directed against membrane receptors or ion channels and playing a pathogenic role has challenged this concept. In case of antibodies targeting intracellular onconeural antigens, patients almost always harbor a tumor; some tumors might be found several years after the onset of neurological symptoms. However, it is not the case in the patients with antibodies targeting surface antigens (ion channels, receptors, or receptor associated proteins).

The reported incidence of PNS varies greatly since most estimates are from referral centers and not from population-based studies [2]. Paraneoplastic sensory neuropathy is probably the most common (3–7 per 1000 cancer diagnoses),

followed closely by paraneoplastic encephalitis (3 per 1000) and cerebellar degeneration (2 per 1000) [3]. A rough classification of PNS is illustrated in Table 1 [4].

2. Limbic Encephalitis: An Increasingly Recognized Entity Belonging to PNS

The limbic system of brain comprises hippocampus, amygdala, hypothalamus, corpus mamillare, fornix, and gyrus cinguli (the Papez circuit) and is responsible for cognition, affect, and autonomic regulation. Limbic encephalitis was described for the first time by Brierley and colleagues in 1960 [5]. It is characterized by subacute onset (from days to several months) of short-term memory loss, disorientation, seizures, confusion, behavioral disturbance, psychiatric symptoms, and altered consciousness suggestive of involvement of the limbic system [6]. Less frequently, patients can have delusional thoughts and paranoid ideation [7], and some patients may have hyponatremia.

In the last decades, limbic encephalitis has been extensively investigated. According to the current knowledge, all types of limbic encephalitis fall into one of two main categories, infectious or autoimmune etiology. Infectious limbic encephalitis is caused by direct invasion of the brain by infectious agents, usually viruses, whereas autoimmune limbic encephalitis is caused by the individual's autoimmune reaction against itself. The current review will center on autoimmune limbic encephalitis and its clinical characteristics. Of note is that although the etiology was historically considered paraneoplastic, limbic encephalitis may also arise from nonparaneoplastic mechanisms, that is, autoimmune processes independent of malignancy. The clinical presentations are quite similar in the two groups. Prodromal flu-like symptoms may point to a nonparaneoplastic etiology, whereas smoking and weight loss suggest a paraneoplastic etiology [8]. The difficulty in differentiating the two categories stems from the fact that in 60% to 70% of paraneoplastic cases, neurological symptoms precede the detection of the tumor [9, 10].

Established diagnosis of this syndrome after exclusion of infective and toxic disorders should prompt the initiation of immunotherapy [11]. The following investigations may aid an accurate diagnosis: analysis of cerebrospinal fluid (CSF), electroencephalogram (EEG), magnetic resonance imaging (MRI), positron emission tomography (PET), and detection of onconeural antibodies in the CSF and/or serum. CSF usually shows lymphocytic pleocytosis, increased protein concentration, and oligoclonal bands. Regardless of the type of clinical presentation, EEG is almost always abnormal, typically revealing focal or generalized slow wave abnormalities or epileptic form discharges in the temporal lobes [12]; T2-weighted or fluid-attenuated inversion recovery (FLAIR) MRI may show hyperintense signals of the medial temporal lobes, although other sites of lesions can also be detected (Figure 1); ^{18}F-fluorodeoxyglucose (FDG) positron emission tomography (PET) may detect hypermetabolism in the medial temporal lobes, even when MRI is normal [12]; various antibodies may be present in serum and CSF. The information provided by the combination of clinical, EEG, MRI, and CSF routine studies suggests the diagnosis of limbic encephalitis in most patients with a classic presentation of the syndrome [12]. It is not mandatory that all investigations show pathological features, and not all cases of limbic encephalitis have typical MRI findings. However, if EEG, MRI, and CSF analyses are all normal, the diagnosis of limbic encephalitis is highly unlikely [8]. The diagnostic criteria for limbic encephalitis are listed in Table 2 [13], and the differential diagnoses of limbic encephalitis are summarized in Table 3.

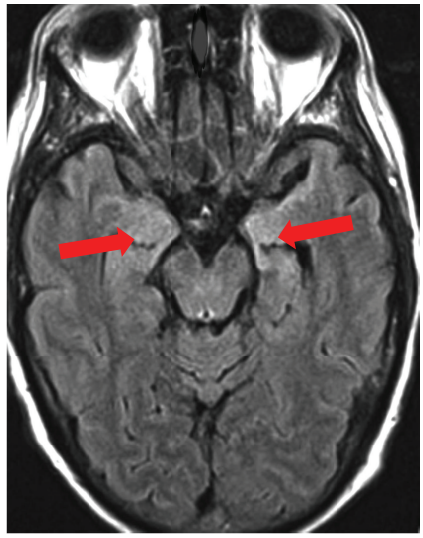

Figure 1: MRI FLAIR of a patient with limbic encephalitis and positive NMDAR antibodies in the CSF. Increased signal intensity is seen in the bilateral medial temporal lobes and hippocampi.

Clinical characteristics of the different types of limbic encephalitis significantly vary according to the antibody type. NMDAR encephalitis often presents with cognitive and behavioral abnormalities. Because psychiatric symptoms are early and prominent, it is not rare for patients to be treated with antipsychotic drugs at onset. Subsequently, characteristic features develop, including movement disorders (orofacial dyskinesia, dystonia), seizures, speech disorder, autonomic dysfunction, central hypoventilation, catatonia, and depressed level of consciousness [8, 11]. Patients with PNS and LGI1-antibodies usually present with classic limbic encephalitis but may show some specific features, such as hyponatremia, rapid eye movement (REM), sleep behavioral disorders, or characteristic tonic seizures. Factually, the concept that limbic encephalitis is an inflammatory disorder strictly limited to anatomic regions of the limbic system is inaccurate. In this regard, these nonrestricted inflammatory boundaries are the rule rather than the exception, particularly when the limbic encephalitis is paraneoplastic. This is evidenced by many pathologic studies that have shown inflammatory infiltration distant from the limbic system. In these patients, a careful clinical evaluation almost always reveals signs of involvement of other areas of the nervous system that may remain mild or become more prominent than the symptoms of limbic dysfunction. For example, PNS in

Table 1: Classification of paraneoplastic neurological syndromes.

Central nervous system
Limbic encephalitis
Encephalomyelitis
Brainstem encephalitis
Stiff-person syndrome
Opsoclonus-myoclonus
Subacute cerebellar degeneration
Paraneoplastic visual syndromes
Cancer-associated retinopathy
Melanoma-associated retinopathy
Paraneoplastic optic neuropathy
Motor neuron syndromes
Subacute motor neuronopathy
Other motor neuron syndromes
Peripheral nervous system
Acute sensorimotor neuropathy
Subacute sensory neuronopathy
Chronic sensorimotor neuropathy
Subacute autonomic neuropathy
Paraneoplastic peripheral nerve vasculitis
Neuromuscular junction and muscle
Myasthenia gravis
Lambert-Eaton syndrome
Polymyositis/dermatomyositis
Acute necrotizing myopathy
Cachectic myopathy
Neuromyotonia

many patients with anti-Hu antibodies may start as limbic encephalitis that often evolves to encephalomyelitis with dorsal root ganglionitis.

3. Tumors That Are Associated with Limbic Encephalitis

In PNS, 50% to 80% of the patients present with neurological symptoms of PNS prior to diagnosis of tumors [15]. The associated tumors in PNS are a lung cancer in 50–60% of patients, usually SCLC (40–55%), and the associated tumor is a testicular germ cell tumor in 20% of patients. Other associated tumors include breast cancer, thymoma, Hodgkin's lymphoma, and teratomas [2]. In paraneoplastic limbic encephalitis, the most common tumors and corresponding antibodies are SCLC (anti-Hu, anti-CRMP5, and anti-amphiphysin), testicular cancer (anti-Ma2), thymoma (anti-CRMP5), and breast cancer (anti-amphiphysin) [16]. In men younger than the age of 50 years with anti-Ma2 antibodies, limbic encephalitis is almost always associated with testicular germ cell tumors, which however can be microscopic and difficult to detect.

As one of the classical PNS, limbic encephalitis can be diagnosed within less than 5 years before cancer is detected [14]. Removal of the tumor is critical for neurologic improvement or stabilization of symptoms in PNS. Therefore, tumor should be screened in patients with limbic encephalitis.

4. Antibodies Commonly Detected in Limbic Encephalitis

Tumor immunologists introduced the term "onconeural" antibodies to designate antibodies that target antigens present in neuroectodermal tissues and tumors [17]. These antibodies are unambiguously demonstrated by standardized tests, associated with limited subsets of tumors, and are present in several PNS types [1]. Since the 1980s, various onconeural antibodies have been discovered, which can serve as biomarkers for classic paraneoplastic syndromes [18]. Classical limbic encephalitides with temporal lobe seizures are associated with onconeural antibodies directed against the intracellular antigens. Onconeural antibodies are found in about 60% of the patients with paraneoplastic limbic encephalitis. The most frequent related antibodies are anti-Hu, anti-Ma2 (with or without Ma1), anti-amphiphysin, and anti-CRMP5. The majority of patients with anti-Hu antibodies have symptoms also suggestive of the dysfunction of areas of the nervous system outside the limbic system.

In recent years, the spectrum of chronic inflammatory brain diseases characterized by the presence of antigen-specific antibodies in serum and CSF has greatly expanded. Many patients with paraneoplastic limbic encephalitis previously characterized as "seronegative" have in fact antibodies against cell surface antigens. Recent studies indicated that most cases previously considered "seronegative" have, in fact, antibodies against surface antigens [19]. More and more cases such as glutamic acid decarboxylase (GAD) 65 antibody encephalitis [20], the voltage-gated potassium channel (VGKC) complex antibody encephalitis [21] (including LGI1 and Caspr2 antibodies), N-methyl-D-aspartate receptor (NMDAR) antibody encephalitis [22], alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) [23], and gamma aminobutyric acid receptor GABA(B) antibody encephalitis [24] are recognized. In a few years, the number of onconeural antibodies described in PNS has increased dramatically. Antibodies to the components of VGKCs, NMDARs, AMPARs, GABA(B), mGluR5 receptor, and glycine receptors (GlyRs) can be identified in patients and are associated with various clinical presentations, such as limbic encephalitis and complex and diffuse encephalopathies [23, 25, 26]. These diseases can be associated with tumors, but some of them are nonparaneoplastic, and antibody assays can help with the diagnosis. The identification of these new antibodies (cell surface antigen associated) has allowed recognition of a syndrome with clinical and radiological features indistinguishable from "classic limbic encephalitis." The course of the newly identified syndromes tends to be less severe and it is often possible to achieve complete recovery with prompt immunomodulatory treatment. The most representative condition is LGI1-encephalitis, previously known as limbic encephalitis with VGKC complex antibodies [27, 28].

Table 2: Diagnostic criteria of paraneoplastic limbic encephalitis.

Criteria by Gultekin et al. [13]
Pathological demonstration of limbic encephalitis, or all 4 of the following.
(1) Symptoms of short-term memory loss, seizures, or psychiatric symptoms suggesting involvement of the limbic system
(2) <4 yr between the onset of neurological symptoms and the cancer diagnosis
(3) Exclusion of metastasis, infection, metabolic and nutritional deficits, stroke, and side-effects of therapy that may cause limbic encephalopathy
(4) At least one of the following:
(a) CSF with inflammatory findings
(b) MRI FLAIR or T2 unilateral or bilateral temporal lobe hyperintensities
(c) EEG with epileptic or slow activity focally involving the temporal lobes
Criteria by the Paraneoplastic Neurological Syndrome Euronetwork [14]
All 4 of the following items are met.
(i) Subacute onset (days or up to 12 wk) of seizures, short-term memory loss, confusion, and psychiatric symptoms
(ii) Neuropathologic or radiologic evidence (MRI, SPECT, PET) of involvement of the limbic system
(iii) Exclusion of other possible etiologies of limbic dysfunction
(iv) Demonstration of a cancer within 5 yr of the diagnosis of neurologic symptoms or the development of classic symptoms of limbic dysfunction in association with a well-characterized paraneoplastic antibody (Hu, Ma2, CRMP5, amphiphysin, Ri)

These antibodies are directed against two categories of antigens: (1) intracellular antigens (Hu, Ma2, CRMP5, amphiphysin, etc.) and (2) cell surface antigens (the VGKC complex, NMDAR, AMPARs, GABABRs, mGluR5 receptor, GlyRs, etc.). Whereas the disorders related to the first category of antibodies are associated with cancer (lung, testis, etc.), prominent brain infiltrates of cytotoxic T cells, and limited response to treatment, the disorders related to the second category of antibodies are associated less frequently with cancer (thymoma, teratoma), seem to be antibody mediated, and respond significantly better to immunotherapy. These two antibodies have in common the association with idiopathic or paraneoplastic limbic encephalitis [23, 24]. Seven out of 15 (47%) patients with limbic encephalitis associated with GABA (B) receptor antibodies had an underlying tumor, usually an SCLC [24]. In limbic encephalitis associated with AMPAR antibodies, the frequency of cancer was 64%, with SCLC being the most common type, followed by thymoma and breast cancer [23]. These patients have a better prognosis than those with antibodies against intracellular proteins [29, 30]. Table 4 summarizes the common antibodies against onconeural antigens detected in PNS and their potentially associated tumors.

5. Do Antibodies Play a Pathogenic Role in Limbic Encephalitis?

A cancer-stimulated immune response that cross-reacts with neural tissue—onconeural immunity—is considered the principal pathologic mechanism for PNS [31]. Some cancer cells express proteins that are normally restricted to the nervous system. For example, when serum from a patient with limbic encephalitis was incubated with the patient's cancer cells and with a rat's brain tissue, antibody fixation to the same Ma proteins on both neurons and cancer cells could be observed [31]. Pathological examination of the nervous system showed loss of neurons in affected areas of the nervous system with inflammatory infiltration by CD4+ T helper cells and B cells in the perivascular spaces and cytotoxic CD8+ T cells in the interstitial spaces [32–34]. Examination of CSF frequently demonstrates pleocytosis, intrathecal synthesis of IgG, and oligoclonal bands, supporting an inflammatory or immune-mediated etiology.

The discovery of paraneoplastic antineuronal antibodies resulted in the general belief that these are immune-mediated disorders triggered by onconeural antigens expressed by tumor cells. Despite the clear clinical evidence that many of the syndromes described earlier are antibody mediated, there is lack of direct evidence showing that these antibodies are pathogenic in PNS. Support for a pathogenic role of antibodies comes from the fact that the target paraneoplastic antigens are expressed both in the tumors and in the affected regions of the nervous system. Furthermore, the size of tumors is usually small and they are heavily infiltrated with inflammatory cells. Interestingly, spontaneous remissions of carcinoma may occur at the time of neurological presentation [35, 36]. One study even found more limited disease distribution and better oncologic outcome in SCLC patients with paraneoplastic antibodies [37].

There are studies on the effects of the serum or CSF IgG antibodies on the neuronal function in cultured cells [22, 23, 38] or on brain slices, but the transfer of clinical or electrophysiological evidence of disease to experimental animals by either systemic or intrathecal injection has not yet been reported, with the exception of mGluR1-Ab in paraneoplastic cerebellar degeneration [39] and reports of GAD-65 or amphiphysin antibodies [40, 41]. In some PNSs, circumstantial evidence suggests that T-cell-mediated mechanisms play a major pathogenic role [42]. It has been suggested that the most important determinant of the underlying immunopathogenesis and responsiveness to immunosuppression is the antibody type and level of the affected individual, which may determine the response to treatment [1, 18, 43].

Table 3: Differential diagnoses of limbic encephalitis.

Infectious disorders
Herpes simplex virus encephalitis
Neurosyphilis
Progressive multifocal leukoencephalopathy
Rabies
Creutzfeldt-Jakob disease
Metabolic disorders
Metabolic encephalopathy (uremic, hepatic, Cushing syndrome, etc.)
Wernicke-Korsakoff syndrome
Hashimoto's encephalopathy
Systemic autoimmune disorders
Sjögren syndrome
Systemic lupus erythematosus
Antiphospholipid syndrome
Malignancies
Lymphoma
Glioma
Gliomatosis cerebri
Degenerative disorders
Alzheimer's disease
Lewy-body dementia
Frontotemporal dementia
Others
Stroke with posterior cerebral artery involvement
Central nervous system vasculitis
Temporal lobe epilepsy
Nonconvulsive status epilepticus
Transient global amnesia
Acute demyelinating encephalomyelitis
Posterior reversible encephalopathy syndrome
Intoxication (alcohol, lithium, etc.)
Alcohol withdrawal syndrome
Psychiatric disorder

Specifically, striking differences have been found between disorders with antibodies against intracellular antigens versus those to neural surface antigens. Disorders with antibodies to intracellular antigens are considered poorly responsive to immunotherapy [18, 20] and may be mediated by cytotoxic T cells [18, 34]. On the other hand, disorders associated with antibodies against cell surface antigens, such as the VGKC-complex or NMDAR, often respond well to treatment [20, 44].

Some laboratory evidence supports the role of pathogenic B-cell responses in limbic encephalitis. NMDAR antibodies from patients have been shown to decrease the numbers of NMDAR in postsynaptic dendrites of cultured hippocampal neurons. One study suggested that anti-Hu antibodies induced apoptosis when applied to cultures of neuroblastoma or mesenteric cells [45]. There is also evidence, however, pointing to that paraneoplastic limbic encephalitis may be T-cell mediated, as Hu-specific T cells have been found in the blood and CSF [46], and there are cytotoxic infiltrates of T cells in the brain and tumor of the patients with anti-Hu antibodies-associated encephalomyelitis [47].

A pathogenic role could only be proven for those paraneoplastic antibodies that are directed against easily accessible antigens located on the cell surface. In these disorders, indirect lines of evidence support the view that the cellular immune responses against these antigens are responsible for the neurological damage [46, 48, 49]. The relative contribution of the cellular and humoral immunity to the clinical and pathological manifestations has not been displayed. The paraneoplastic antibodies may, in these cases, be surrogate markers for T-cell activation [50]. Elevated CD8/CD3 ratios in diseases were associated with antibodies to intracellular antigens and suggested a cytotoxic T-cell-driven pathomechanism. In diseases with antibodies to surface antigens, this finding supports a B-cell-related pathomechanism, with evidence of a complement-mediated pathogenesis in patients with VGKC-complex antibodies. Interestingly, this immunopathogenic dichotomy parallels other autoimmune disorders such as polymyositis and dermatomyositis, which have a predominant T-cell- and antibody-mediated pathogenesis, respectively [51]. These observations may contribute to a rational choice in immunotherapies for these disorders [52]. A totally different mechanism seems at work in paraneoplastic cerebellar degeneration in Hodgkin's lymphoma because the target antigens of the associated anti-Tr and anti-mGluR1 antibodies are not expressed in Hodgkin's tumor tissue [53]. Dysregulation of the immune response in Hodgkin's lymphoma and an etiologic role for viral infections have been postulated in this disorder [53].

Thus far, it is still unclear whether antibody-mediated PNS, for example, VGKC complex antibody-associated limbic encephalitis, is driven by serum or intrathecal antibodies. The absolute concentrations of antibodies against a certain onconeural antigen are usually higher in serum than in the CSF. Moreover, antibodies are not always detectable in the CSF. Ideally, both serum and CSF samples should be sent for antibody testing, but their relative utility in followup of patients is under debate. Intrathecal synthesis of IgG and oligoclonal bands can help pointing to an immune-mediated disorder before the results of specific antibodies can be obtained, but the oligoclonal bands are not always present at onset or even thereafter, and whether their presence is evidence for ongoing pathology or merely a secondary epiphenomenon is not yet clear. The intrathecal synthesis of antibodies can actually be assessed by the calculation of the amount of specific antibodies in the CSF relative to the total CSF IgG and by comparison with similar calculations in the serum. The ratio represents intrathecal synthesis and is often higher in some PNS. In favor of a role for systemic rather than intrathecal antibodies, animal experiments have shown that certain regions of the brain, that is, the hippocampus

Table 4: The common antibodies detected in PNS and their associated tumors.

Antibodies	PNS	Associated tumors
	Antibodies against intracellular antigens	
Anti-Hu	sensory neuronopathy, LE, BSE, encephalomyelitis	SCLC
Anti-Yo	SCD	Gynecological cancer
Anti-Ri	Opsoclonus-myoclonus, BSE	Breast cancer
Anti-Ma2	BSE, LE	Testis cancer, SCLC, breast cancer
Anti-CRMP5	SCD, chorea, myelitis, LE, sensory neuronopathy, optic neuritis	SCLC, thymoma
Anti-amphiphysin	SPS, myelitis, SCD, sensory neuronopathy	SCLC, breast cancer
Anti-GAD-65	SPS, myelitis	SCLC, breast cancer
Anti-SOX-1	LEMS	SCLC
	Antibodies against cell surface onconeural antigens	
Anti-VGCC	SPS, LEMS	SCLC
Anti-VGKC complex	LE	SCLC, thymoma
Anti-NMDA receptor	LE	Teratoma
Anti-AMPA receptor	LE	SCLC, breast cancer, thymoma
Anti-AQP-4	NMO spectrum disorders	SCLC, breast cancer, thymoma
Anti-GABA-B receptor	LE	SCLC
Anti-CAR	Retinopathy	SCLC, melanoma, gynecological cancer
Anti-contactin-associated protein 2	Morvan syndrome	Thymoma
Anti-AchR/MuSK/RyR/Titin	MG	Thymoma

AMPA: amino-3-hydroxyl-5-methyl-4-isoxazole-propionate; AQP-4: aquaporin 4; CAR: cancer-associated retinopathy; CRMP5: collapsin response mediator protein 5; GABA-B: gamma-aminobutyric acid B; GAD-65: glutamic acid decarboxylase 65; LE: limbic encephalitis; LEMS: Lambert-Eaton myasthenic syndrome; MG: myasthenia gravis; NMDA: N-methyl-D-aspartate; NMO: neuromyelitis optica; SCD: subacute cerebellar degeneration; SCLC: small cell lung cancer; SPS: stiff-person syndrome; VGCC: voltage-gated calcium channel; VGKC: voltage-gated potassium channel.

and the hypothalamus, seem to be particularly vulnerable, and it is notable that limbic encephalitis with VGKC-complex (LGI1 and Caspr2) antibodies and anti-NMDAR encephalitis usually start with symptoms originating from the temporal lobe cortex, even though the target antigens are present much more widely in the CNS. The former usually affects hippocampus, amygdala, and anterior temporal cortex, whereas the latter usually affects hippocampus, cerebral cortex, basal ganglia, and thalamus [54]. Until recently, only 50% of patients with limbic encephalitis and SCLC were found antibody positive, usually harboring anti-Hu antibodies or, less frequently, other onconeural antibodies [29].

Immunopathological analysis of various antibody-associated limbic encephalitis may help elucidate the underlying immunopathogenic mechanisms, whereas unfortunately there has been a lack of laboratory data [52]. Why is limbic encephalitis reversible in patients with NMDAR antibodies that are in frequent association with ovarian teratoma [44, 55]? Furthermore, how does one classify those patients with GAD-65 antibodies that are not paraneoplastic in origin, who suffer from limbic encephalitis or chronic temporal lobe epilepsy [20]?

An important issue is that a positive report for any well-characterized onconeural antibody has to be assessed according to the clinical setting. All these antibodies, particularly those associated with SCLC, can be found in the patients with cancer without PNS [56]. Therefore, one should still rule out other potential causes of the neurological syndrome that is being evaluated. Up to 16% of patients with SCLC without PNS have low titers of Hu antibodies, whereas in the patients with PNS and Hu antibodies, the titers are substantially higher [37].

6. Treatments of Limbic Encephalitis

The basic principles of paraneoplastic limbic encephalitis therapy are resection of the tumor or oncological treatment [10]. When a patient with tumor is found in association with a possible paraneoplastic disorder, removal of the tumor is critical for neurologic improvement or stabilization of symptoms. Antibodies against onconeural antigens are sensitive and should prompt an extensive tumor screening in antibody-positive patients. In the patients with limbic encephalitis associated with ion channel/receptor antibodies, immunosuppressive or immunomodulatory treatment is promising to improve the disease. Limbic encephalitides with antibodies against intracellular onconeural antigens do not normally respond to immunosuppressive treatment; only tumor therapy may stabilize the syndrome. Treatment of PNS still remains difficult. Anti-Hu-antibody-positive patients do not normally respond to immunosuppressive treatment. The only therapy that stabilizes these patients is perhaps the

Table 5: Immunotherapies for limbic encephalitis.

Acute phase of the disease
First-line therapies
High-dose corticosteroids
Intravenous immunoglobulins
Plasma exchange
Second-line therapies
Rituximab
Cyclophosphamide
Maintenance therapy
Steroids
Azathioprine
Mycophenolate

tumor treatment itself [16, 57]. In other PNSs associated with defined onconeural antibodies, only a few patients have beneficial effects after treatment [58]. Most patients with limbic encephalitis and ion channel receptor antibodies also respond to immunosuppressive or immunomodulatory treatment [22, 23, 27, 59]. Patients with limbic encephalitis and antibodies against cell surface antigens such as VGKC or NMDAR often respond to immunotherapies, such as corticosteroids, intravenous immunoglobulin (IVIG), or plasma exchange. Other therapy regimens that might be of relevance are rituximab, cyclophosphamide, and azathioprine [60]. In limbic encephalitis patients with intracellular antibodies, Ma2-positive patients may respond better to immunosuppression than patients with anti-Hu or anti-CRMP5 antibodies [61]. Immunotherapies for limbic encephalitis have been summarized in Table 5.

7. Some Conclusions and More Questions

This field of immune-mediated CNS diseases is exciting but also challenging. Ideally, antibody testing should be performed using internationally validated procedures so that the diagnosis can be made and treatments started as soon as possible in the hope of restoring health, limiting hospitalization, and optimizing outcomes. Systematic studies of the treatments are needed in order to establish the best practice. Experience with the recently described antibodies, with exception of those against NMDAR, is still relatively small. Therefore, their inclusion in one particular group of the proposed classification must be viewed with caution until more cases are described. Good clinical-immunological correlations are crucial to define the clinical syndrome that most likely associates with a particular antibody.

The so-far identified antibodies might only be the tip of the iceberg, with antibodies to other membrane ion channels or receptors awaiting recognition in future. Even now, the range of clinical features exhibited by patients with VGKC, NMDAR, aquaporin (AQP) 4, or GlyR antibodies is wide and includes most aspects of the nervous system. Researchers in this field must provide good clinical descriptions of the case series associated with the antibodies they study. This approach will help clinicians identify the clinical syndromes and make a rational decision on which antibodies to request. Moreover, more effective methods are required for the detection of onconeural antibodies [62].

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Research Article

Systemic Immune Activation Leads to Neuroinflammation and Sickness Behavior in Mice

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Substantial evidence indicates an association between clinical depression and altered immune function. Systemic administration of bacterial lipopolysaccharide (LPS) is commonly used to study inflammation-associated behavioral changes in rodents. In these experiments, we tested the hypothesis that peripheral immune activation leads to neuroinflammation and depressive-like behavior in mice. We report that systemic administration of LPS induced astrocyte activation in transgenic GFAP-luc mice and increased immunoreactivity against the microglial marker ionized calcium-binding adapter molecule 1 in the dentate gyrus of wild-type mice. Furthermore, LPS treatment caused a strong but transient increase in cytokine levels in the serum and brain. In addition to studying LPS-induced neuroinflammation, we tested whether sickness could be separated from depressive-like behavior by evaluating LPS-treated mice in a panel of behavioral paradigms. Our behavioral data indicate that systemic LPS administration caused sickness and mild depressive-like behavior. However, due to the overlapping time course and mild effects on depression-related behavior per se, it was not possible to separate sickness from depressive-like behavior in the present rodent model.

1. Introduction

Clinical depression is a devastating, recurrent psychiatric illness that has a lifetime prevalence of 16% [1]. By the year 2030, depression is predicted to become the second leading cause of disability worldwide [2]. Despite its high prevalence and considerable socioeconomic impact, very little is known about the pathophysiology of the disorder. Increasing numbers of studies support the idea that depression is a multifactorial disease with both genetic and environmental factors contributing to disease development [3]. Inflammatory processes may also play a role in the etiology of depression, at least in a subset of susceptible individuals. It has been reported that depressed patients commonly display alterations in their immune system, including impaired cellular immunity and increased levels of proinflammatory cytokines; for reviews, see Schiepers et al. 2005 [4], Dowlati et al. 2010 [5], Blume et al. 2011 [6], and Howren et al. 2009 [7]. Furthermore, depression frequently occurs as a comorbidity of conditions that are characterized by a sustained, systemic inflammation such as rheumatoid arthritis [8, 9], coronary heart disease [10, 11], stroke [12], type 2 diabetes [13], and obesity [14]. Another indication that inflammation and depression are linked comes from clinical observations in which therapeutic administration of the proinflammatory cytokines interleukin-2 and interferon-α to cancer or hepatitis C patients resulted in depression in up to half of these patients [15–17].

Bacterial lipopolysaccharide (LPS) is a potent activator of the immune system. Numerous reports have shown that systemic administration of LPS in animals leads to sickness, a behavioral state characterized by symptoms including lethargy, decreased locomotor activity and appetite, anhedonia (the inability to experience pleasure from naturally

rewarding activities), sleep disturbances, and increased sensitivity to pain [18, 19]. Several of these symptoms are thought to be very similar to clinically relevant symptoms of depression in humans [20, 21]. Therefore, systemic administration of LPS is frequently used to study inflammation-associated depression in rodents. Some rodent studies report that, 24 h after systemic LPS injection, depressive-like behavior is present without the confounding effects of sickness [22–24]. However, these findings are not consistent across the literature, with some studies describing depressive-like behavior at earlier time points [25, 26] and others still reporting signs of sickness at 24 h [27–30]. Moreover, studies focusing on LPS-induced depressive-like behavior often vary in LPS dose, LPS serotype, application route, and assays used, which makes it difficult to compare results between research groups. In addition, many of these studies only use a single dose of LPS and/or a few time points, thus making it impossible to assess time- and dose-dependent changes in neuroinflammation and behavior.

The present study aimed at evaluating central effects of peripheral immune activation by combining multiple techniques to quantify neuroinflammation and behavioral changes at several time points after systemic LPS administration. First, transgenic GFAP-luc mice were used to assess the kinetics of LPS-induced astrocyte activation, as marker of neuroinflammation. After confirming the presence of neuroinflammation by immunohistochemistry using the microglial marker ionized calcium-binding adapter molecule 1 (IBA1), serum and brain levels of immune mediators were measured at time points corresponding to glial activation. Finally, LPS-treated mice were tested in a panel of behavioral paradigms to evaluate whether depressive-like behavior could be separated over time from sickness.

2. Material and Methods

2.1. Animals and LPS. Male NMRI mice were obtained from Charles River Laboratories (France), male wild-type FVB mice from Janvier (France), and GFAP-luc transgenic mice (FVB/N-Tg(Gfap-luc)-Xen) were purchased from Taconic Laboratories (USA). These latter animals express the firefly luciferase gene under the control of a 12 kb murine glial fibrillary acidic protein (GFAP) promoter [31] and are commonly used to noninvasively measure astrocyte activation in the same animal over time [31–36]. Unless mentioned otherwise, animals were housed in groups of 4 in individually ventilated cages (IVC; L × W × H: 36 × 20 × 13 cm; Tecniplast, Italy) under a normal 12:12 h light-dark cycle (lights on at 06:00 a.m. with a 30 min dim and rise phase). Procedure rooms were maintained at a temperature of 22 ± 2°C and a humidity of 54 ± 2%. Food and water were available *ad libitum*. All experimental protocols were approved by the Institutional Ethical Committee on Animal Experimentation, in compliance with Belgian law (Royal Decree on the protection of laboratory animals dd. April 6, 2010) and conducted in facilities accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC).

Lipopolysaccharide (LPS) from *Escherichia coli* (serotype 055:B5) was purchased from Sigma-Aldrich and freshly dissolved in sterile saline prior to injection.

2.2. Bioluminescence. Astrocyte activation in 10-week-old male GFAP-luc mice was monitored before (baseline) and at specific time points (2 h, 6 h, 24 h, 48 h, 72 h, and 96 h) after intraperitoneal (i.p.) administration of either 0, 0.16, or 0.63 mg/kg LPS. Results from a pilot experiment showed that a dose of 2.5 mg/kg LPS in combination with the experimental procedure to measure bioluminescence was lethal in GFAP-luc mice. Therefore, it was decided to use 0.63 mg/kg LPS as the highest dose in this experiment.

To detect the bioluminescent signal, mice were anesthetized by inhalation of 2% isoflurane, shaved on the head, and injected with 126 mg/kg D-luciferin (Promega, product ID E1601) in the tail vein. Three minutes later, the animals were scanned with a charge-coupled device (CCD) camera (IVIS Imaging System 200 Series, Xenogen) mounted on a dark box. The imaging signal was measured in physical units of surface radiance (photons/s/cm^2/steradian [sr]) using Living Image 3.2 software (Xenogen). Photon emission from the brain was counted using a region of interest (ROI) that was kept constant within the experiment. Bioluminescence coming from the ear was considered to be basal GFAP activity and was excluded from the ROI.

2.3. Immunohistochemistry. 10-week-old male FVB mice were injected i.p. with vehicle or 0.63 mg/kg LPS, and tissue was collected for immunohistochemical staining 24 h later. Mice were anesthetized with 60 mg/kg sodium pentobarbital (Nembutal) and transcardially perfused with 25 mL heparinised 0.9% saline followed by 50 mL 4% paraformaldehyde (PFA) in 1x phosphate-buffered saline (PBS) (pH 7.4, 4°C). Brains were dissected and postfixed in 4% PFA overnight at 4°C, before being washed twice in PBS and stored in PBS/0.1% NaN3 at 4°C. Free-floating coronal brain sections of 100 μm thickness were cut at the level of the hippocampus (Interaural 1.50 mm, Bregma −2.30 mm, Paxino & Watson, 2001) using a Leica VT1000S vibratome (Leica Microsystems) and were subsequently stored in PBS/0.1% NaN3 at 4°C until use.

For the immunofluorescent staining of IBA1 protein, sections were washed 3 × 5 min in PBS before being incubated in blocking buffer (5% goat serum, 0.3% Triton X 100, and 0.1% bovine specific albumin (BSA) in PBS) for 3 h. Subsequently, sections were incubated overnight at 4°C with a rabbit polyclonal anti-IBA1 (1 : 500, Wako) primary antibody in blocking buffer. The following day, sections were washed 3 × 5 min in PBS before being incubated in PBS-BSA containing the secondary fluorescent antibodies Alexa 555 goat anti-rabbit (1 : 500, Invitrogen), for 2 h at room temperature in the dark. After 3 × 5 min washes in PBS, sections were mounted onto glass slides using a glycerol-based mounting medium containing DABCO (100 mg/mL) and stored in the dark.

A confocal scanning Zeiss Axiovert 100 M microscope was used to obtain fluorescent images. Single images were captured using a Zeiss Plan-Neofluar 10x (NA 0.30) lens.

For each animal, two brain sections were analysed, and fluorescent images containing immunopositive cells at the level of the hippocampal dentate gyrus were captured from the 555 nm wavelength. Image analysis software from Zeiss (LSM 510) was used in order to detect changes in the quantity of IBA1 staining levels. Thresholding was used to distinguish positive cells from background. A boundary was drawn around the dentate gyrus of the hippocampus to exclude other regions from quantification. The output of the analysis was "number of pixels."

2.4. Cytokine Measurements. Based on the course of neuroinflammation seen in GFAP-luc mice, it was decided to measure cytokine levels in serum and brain at 2 h, 6 h, and 24 h after LPS administration. For this purpose, 10-week-old male NMRI mice were injected i.p. with 0, 0.63, or 2.5 mg/kg LPS. To reduce animal usage, the 0.16 mg/kg LPS dose was left out as this dose only caused mild GFAP upregulation in the GFAP-luc mice.

At the relevant time points, mice were killed by decapitation, and serum and brain samples were collected. Serum samples were obtained by collecting truncal blood in Vacutainer SST II Advance blood tubes (BD Biosciences, product ID 367955). After being kept for 30 minutes at room temperature, the blood samples were centrifuged (1300 g, 10 min, room temperature), aliquoted, flash-frozen in liquid nitrogen, and stored at −80°C until further use. Within two minutes after decapitation, the brain was isolated from the skull and the hemispheres were separated. They were then weighed, transferred to Tallprep Matrix D tubes (MP Biomedicals, product ID 116973025), flash-frozen in liquid nitrogen, and stored at −80°C until further processing.

A slightly modified protocol adapted from Erickson et al. 2011 [37] was used to extract total protein from brain samples. Briefly, frozen hemispheres were immersed in a 5x volume of extraction buffer (20 mM Tris, 150 mM NaCl, 2 mM EDTA, and 1 mM EGTA) containing a protease inhibitor (Roche, product ID 11873580001) and phosphatase inhibitor cocktail (Roche, product ID 4906837001), and the tissue was homogenized by shaking with a bench top homogenizer (FastPrep-24, MP Biochemicals) for 25 sec. The homogenate was then centrifuged (1000 g, 10 min, 4°C) and supernatant was removed to be centrifuged a second time (20000 g, 40 min, 4°C). Finally, the protein content of each sample was determined using a bicinchoninic acid assay (Sigma-Aldrich), with bovine serum albumin (Sigma-Aldrich, product ID A4503) as a standard.

Concentrations of interferon-γ (IFN-γ), interleukin- (IL-) 1β, IL-6, IL-10, monocyte chemoattractant protein-1 (MCP-1), and tumor necrosis factor-α (TNF-α) were simultaneously determined in serum and brain samples using a mouse cytokine/chemokine magnetic bead panel kit from Merck Millipore. This assay is based on Luminex technology in which magnetic beads with a distinct emitting fluorescence pattern are coated onto capture antibodies specific for individual cytokines. All steps in the assay were conducted according to the manufacturer's instructions. A Bio-Plex 200 System (Bio-Rad) was used to measure the fluorescent signal, and the data was analyzed using Bio-Plex Manager 5.0 software (Bio-Rad) with five-parameter logistic regression curve fitting. Cytokine and chemokine concentrations in brain samples were then normalized to the total protein concentration determined for each sample. Cytokines levels below detection limit were assigned a value equal to the lowest detectable value of that cytokine. Cytokine values outside of the average ±3 times standard deviation range were considered outliers and were excluded from all calculations. This happened for less than 2% of all measured cytokines.

2.5. Behavioral Tests. Behavioral tests were conducted on 10-week-old male NMRI mice. The open field test (OFT), tail suspension test (TST), and forced swim test (FST) setups were custom-made in-house. In all of these paradigms, groups of naïve mice (n = 10/group) were injected i.p. with 0, 0.31, 0.63, or 1.25 mg/kg LPS and tested at either 2 h, 6 h, or 24 h after LPS administration. This dose range of LPS was selected based on results from our previous experiments. The lowest dose of LPS (0.31 mg/kg) was chosen because 0.16 mg/kg LPS only resulted in a mild increase of bioluminescence in the GFAP-luc mice, and it was speculated that a stronger signal was needed to induce behavioral effects. The highest dose of LPS was set to 1.25 mg/kg because 2.5 mg/kg LPS was lethal in the GFAP-luc mice.

The OFT setup consisted of 4 individual arenas (L × W × H: 40 × 40 × 40 cm). Each arena was lit from the top by a lamp producing a light intensity of 800 lux at the bottom. The four arenas allowed testing of four mice at once, while they were separated by nontransparent walls. A video camera with an infrared filter was fixed into the ceiling of each arena, in a way that it covered the entire surface area of that arena. Infrared illumination was provided below the floor of the arenas so mice could be detected and tracked under optimal conditions. Exactly 2 sec after the detection of each individual mouse, tracking of movement was started and performed for 10 min using Noldus EthoVision, version 6.1 (Noldus Information Technology, The Netherlands), with software set up to detect immobility time and distance moved. In this test, exploration behavior of the animal was used to measure locomotor activity.

After single-housing the animals for one day prior to testing, the stress-induced hyperthermia (SIH) paradigm started by measuring the baseline temperature (Temp1). This was done by dipping a rectal probe (Model N9001, Comark Limited, UK) into peanut oil and inserting the probe for 2 cm into the rectum of the mouse while holding the animal in a head-upward position. 15 min later, this procedure was repeated (Temp2) to determine the impact of the mild stress procedure of handling and probe insertion on rectal temperature. In both cases, the rectal probe was kept in place for 15–20 sec in order to standardize stress exposure and reach a stable temperature readout. The mild stress of handling and probe insertion causes a hyperthermic response, and the difference in temperature before and after stress (dT = Temp2 − Temp1) reflects the SIH response. This SIH response is suppressed by anxiolytic drugs and is evaluated as a measure of anxiety [38].

The TST consisted of six individual chambers (2 rows with 3 columns; each chamber L × W × H: 14 × 14 × 19.5 cm).

A 2.5 cm long hook was fixed to the ceiling of each chamber. The paradigm started by wrapping a piece of tape around the distal part of the tail of each mouse (about 2 cm from the tip) and positioning the mouse upside down when the tape is placed over the hook. The six chambers allowed testing of six mice at once, while they were visually separated by nontransparent walls. A video camera was fixed onto a frame in front of the chambers such that it covered the entire surface of the units. Detection contrast was optimized by using black panels behind the white mice. Exactly 2 sec after detection of each mouse separately, the tracking of movement was started and performed for 6 min. Animals were tracked using Noldus EthoVision, version 6.1, with the software set up to detect immobility time and distance moved (based on center point of gravity of the detected surface). In this test, the animal's immobility was evaluated as a measure of "behavioral despair."

The FST setup consisted of four independent cylinders (diameter 11 cm) which were automatically flushed and filled with water (10 cm deep, 24-25 degrees Celsius). The four cylinders allowed testing of four mice at once, while they were separated by nontransparent walls. A video camera with an infrared filter was fixed onto a frame in front of the cylinders such that it covered the entire surface area of all four units. Behind the cylinders, infrared illumination was provided to allow optimal detection and tracking of the mice. Exactly 2 sec after detection of each individual mouse, the tracking of movement was started and performed for 6 min using Noldus EthoVision 6.1 software. Immobility time and distance moved (based on center point of gravity of the detected surface) were detected, and the animal's immobility was evaluated as a measure of "behavioral despair."

In the sucrose preference test (SPT), animals were single-housed in special Plexiglas IVC (L × W × H: 35 × 31 × 16 cm; Tecniplast, Italy) fitted with two 250 mL plexiglass drinking bottles (Tecniplast). Each bottle contained either filtered tap water or a sucrose solution (1, 2, 5, or 10%). The experiment consisted of a *familiarization* and a *test phase*. During the *familiarization phase,* all animals were presented for 24 h with two water-filled bottles (W/W) on day (D) 1 and D3, or one water- and one sucrose-filled bottle (W/S) on D2 and D4. The bottles were removed between 08:00 and 09:00 a.m. each day and weighed using Software Wedge for Windows 1.2 (TAL Technologies). Animals were weighed, and freshly prepared bottles were put into the cages. The amount drunk by a mouse was determined by subtracting the weight of the bottle at the start of the observation period and at the end 24 h later (taking fluid density as 1 g/mL). Total fluid intake was taken as the total change in volume from both bottles combined, while the preference for sucrose was calculated as a percentage of consumed sucrose solution of the total fluid intake. A total fluid intake that was greater than the mean +2x standard deviation was considered to be an invalid measure that probably resulted from leaking bottles. Invalid measures were replaced by the mean of all the bottles either on the relevant side (for W/W) or for either water or sucrose (for W/S). This happened in less than 1% of all bottle measurements. The *test phase* of the experiment started 3 days after the *familiarization phase* by injecting the mice with either vehicle or 0.63 mg/kg i.p. LPS. This dose of LPS was chosen because it had a clear effect on neuroinflammation and sickness behavior in the previous experiments. Immediately after LPS administration, the mice were presented with W/S for 24 h. This procedure was repeated for 3 consecutive days. Total intake volume was evaluated as a primary measure for sickness behavior (reduction versus normal daily intake), while sucrose preference was used as a measure for anhedonia.

2.6. Statistical Analysis. Data were analyzed using SPSS Statistics software (Version 20 for Windows, IBM Inc.). Analysis of variance (ANOVA) or, when appropriate, ANOVA with repeated measure analysis (rmANOVA) was performed. A Greenhouse-Geisser correction epsilon (ε) was used for repeated measures analysis to correct for potential violation of the sphericity assumption [39]. This correction multiplies both the numerator and the denominator degrees of freedom by epsilon, and the significance of the F-ratio is evaluated with the new degrees of freedom, resulting in a more conservative statistical test. When significant, post hoc comparisons were made by using an independent samples t-test with a Bonferroni-corrected P value. Significance was accepted for the ANOVAs and post hoc comparisons when $P < 0.05$. All data are expressed as mean ± standard error of the mean (SEM).

Bioluminescent signals in the GFAP-luc mouse were analyzed by rmANOVA using dose (3 levels: 0, 0.16, and 0.63 mg/kg LPS) as a between-subjects factor and time (7 levels: BL, 2 h, 6 h, 24 h, 48 h, 72 h, and 96 h) as a within-subject factor. Number of pixels in IBA1 positive cells were analyzed by ANOVA using dose (2 levels: 0 and 0.63 mg/kg LPS) as between-subjects factor. Cytokine levels were analyzed by separate ANOVAs for each cytokine with dose (3 levels: 0, 0.63, and 2.5 mg/kg LPS) and time (2 h, 6 h, and 24 h) as between-subjects factor. Distance moved in OFT and immobility time in TST and FST were analyzed using separate ANOVAs with dose (4 levels: 0, 0.31, 0.63, and 1.25 mg/kg LPS) as between-subjects factor. For the SIH procedure, both temperatures (Temp1 and Temp2) were analyzed as a repeated measure and dose (4 levels: 0, 0.31, 0.63, and 1.25 mg/kg LPS) as a between-subjects factor. Total volume intake and sucrose preference in both phases of the SPT were separately analyzed using rmANOVA. In the *familiarization phase*, flavor (2 levels: W/W and W/S) and repeat (2 levels: first test and retest) were used as within-subject factor and treatment group (5 levels: 1%, 2%, 5%, and 10% sucrose/LPS and 10% sucrose/vehicle) as a between-subjects factor. For the *test phase*, treatment group (5 levels: 1%, 2%, 5%, and 10% sucrose/LPS and 10% sucrose/vehicle) was again used as a between-subjects factor and time (3 levels for total volume intake and sucrose preference: D8, D9, and D10) as a within-subject factor.

3. Results

3.1. Effect of Systemic LPS Administration on Brain Bioluminescence in GFAP-luc Mice. Factorial rmANOVA of photons emitted per second in the brain region of interest revealed a significant time × dose interaction ($F(12, 96) = 15.0$,

$P < 0.001$, $\varepsilon = 0.18$). Post hoc analysis showed that, at 6 h after LPS, a strong and brain-specific bioluminescent signal was present in mice treated with 0.63 mg/kg, while, at this time point, a more moderate but still significant signal was evoked in the 0.16 mg/kg LPS group (Figure 1). For both groups, there was still a significant increase in brain bioluminescence at 24 h, but no longer at 48 h after LPS. Bioluminescence coming from the ears did not change during the experiment and was considered to be a background signal.

Because the bioluminescence data revealed a significant LPS-induced GFAP upregulation, it was decided to confirm the presence of glial activation by immunohistochemistry using a microglial marker. Therefore, the expression of IBA1 was quantified in the hippocampal dentate gyrus at 24 h after systemic administration of vehicle or 0.63 mg/kg LPS. This brain structure was chosen because it is associated with stress and depression [40–42] and commonly studied in models of LPS-induced neuroinflammation [43, 44]. Although astrocyte activation in the GFAP-luc mouse peaked at 6 h after LPS, it was decided to quantify IBA1 expression at 24 h as some studies reported depressive-like behavior in the absence of sickness at this time point [23, 24]. Furthermore, astrocyte activation was still increased in LPS-treated mice at 24 h, thereby indicating the relevance of measuring glial activation at this point. Factorial ANOVA indicated a significant effect of dose ($F(1, 18) = 23.9$, $P < 0.001$), and post hoc analysis showed that the pixel number of IBA1 positive cells was significantly higher in mice that received LPS when compared to vehicle-treated mice (Figure 2).

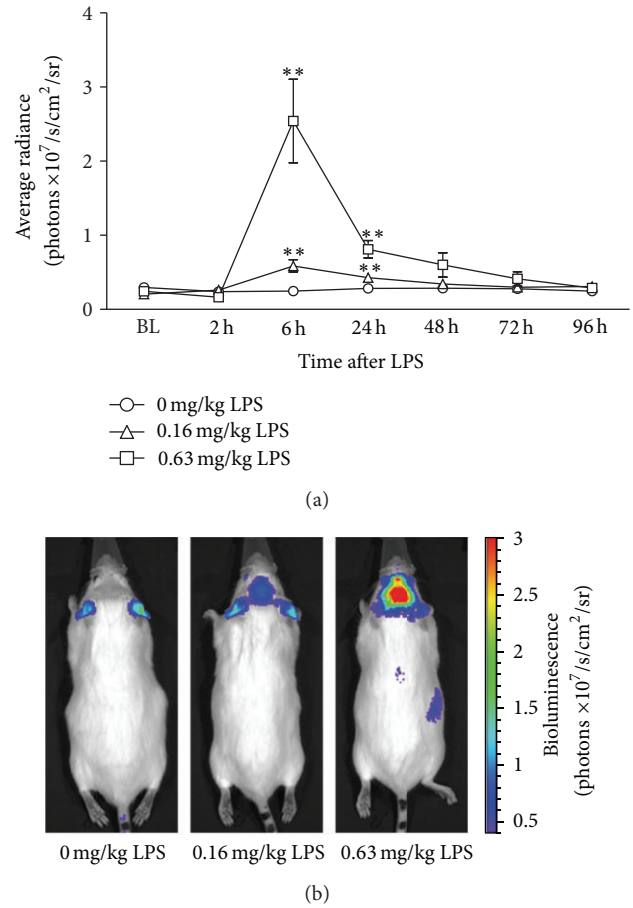


Figure 1: Intraperitoneal administration of LPS caused a dose- and time-dependent increase in brain bioluminescent signal in GFAP-luc transgenic mice (a). A clear LPS-induced bioluminescent signal was visible in the brain, as seen on representative images taken from animals treated with different doses of LPS at 6 hours after injection (b). The color on the image represents the number of photons emitted from the animal per second, as indicated in the color scale on the right. Graphs are plotted as mean + SEM ($n = 8$ per group). Data were analyzed by rmANOVA followed by independent samples t-test. $^{**}P < 0.01$ compared to 0 mg/kg LPS.

3.2. Effect of Systemic LPS Administration on Serum and Brain Cytokine Levels. For all cytokine levels measured in serum, a significant time × dose interaction was found (IL-1β: $F(4, 96) = 6.9$, $P < 0.001$; IL-6: $F(4, 97) = 40.9$, $P < 0.001$; TNF-α: $F(4, 95) = 18.8$, $P < 0.001$; IFN-γ: $F(4, 98) = 4.9$, $P < 0.01$; IL-10: $F(4, 95) = 14.3$, $P < 0.001$; and MCP-1: $F(4, 95) = 22.7$, $P < 0.001$). Post hoc analysis demonstrated that serum cytokine levels in vehicle-treated mice were undetectable or minimal at all time points (Figure 3, left column). Serum levels of IL-1β, IL-6, TNF-α, IL-10, and MCP-1 increased significantly after administration of 0.63 or 2.5 mg/kg LPS, peaking at 2 h after administration and gradually decreasing over time. Serum IFN-γ levels in LPS-treated animals followed a slightly different time course as the peak for this cytokine was reached at 6 h after LPS. At 24 h after LPS administration, the serum levels of IL-1β, TNF-α, and IFN-γ had returned to baseline values, while IL-6 and MCP-1 were still elevated in 0.63 and 2.5 mg/kg LPS-treated animals and IL-10 only in 2.5 mg/kg LPS-treated mice.

A significant time × dose interaction was found on brain levels of IL-1β, IL-6, TNF-α, and MCP-1 (IL-β: $F(4, 98) = 5.6$ $P < 0.05$; IL-6: $F(4, 96) = 9.7$, $P < 0.001$; TNF-α: $F(4, 97) = 8.2$, $P < 0.001$; and MCP-1: $F(4, 97) = 24.3$, $P < 0.001$), but no significant effect of time or dose could be detected on IFN-γ or IL-10 brain levels. Comparable to the time course of their release in serum, brain levels of IL-6, TNF-α, and MCP-1 peaked at 2 h posttreatment in mice exposed to 0.63 and 2.5 mg/kg LPS (Figure 3, right column). Apart from MCP-1 levels, which were still elevated in the brains of LPS-treated mice at 24 h, all brain cytokine levels had returned to baseline values at 24 h after LPS injection. IL-1β was slightly, but significantly, increased at 6 h in the brains of mice that received 2.5 mg/kg LPS, but not at 0.63 mg/kg.

3.3. Effect of Systemic LPS Administration on Behavior across a Panel of Sickness, Anxiety, and Depressive-Like Behavior Assays. The total distance travelled in the open field test is a general measure for exploration and can be used as a marker of sickness behavior. Factorial ANOVA revealed a significant main effect for the factor dose at all time points tested (2 h: $F(3, 36) = 6.6$, $P < 0.01$; 6 h: $F(3, 35) = 23.7$, $P < 0.001$; and 24 h: $F(3, 36) = 4.3$, $P < 0.05$). Post hoc analysis demonstrated that animals exposed to 0.63 or 1.25 mg/kg LPS showed reduced locomotor activity at 2 h posttreatment

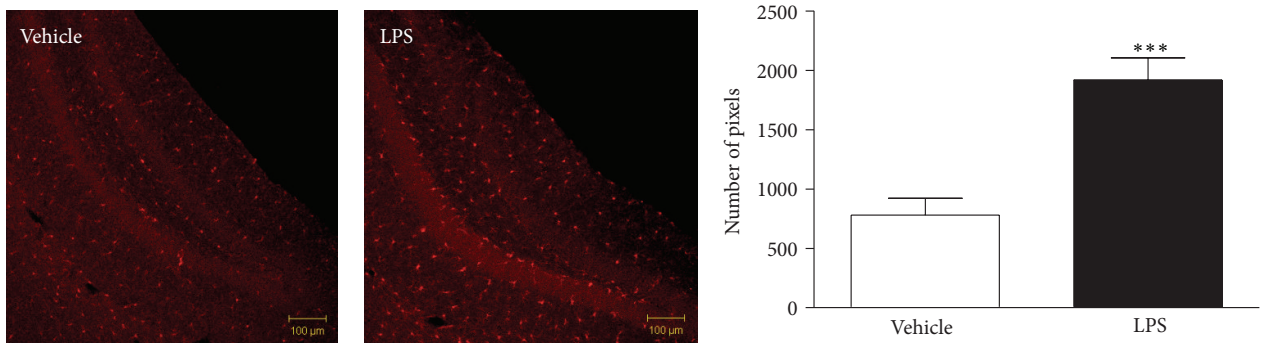


Figure 2: Peripheral LPS injection (0.63 mg/kg, i.p.) increased IBA1 immunoreactivity, a marker of microglial activation, in the hippocampal dentate gyrus at 24 h after administration. Representative images (10x) (a), quantified images of $n = 10$ per group (b). Graph is plotted as mean + SEM. Data were analyzed by ANOVA followed by independent samples t-test. $^{***}P < 0.001$ compared to vehicle.

(Figure 4, OFT). At 6 h, all doses of LPS led to a reduced distance travelled in the OFT, while at 24 h only mice treated with 0.63 or 1.25 mg/kg LPS showed a significant reduction in exploration when compared to vehicle-treated mice.

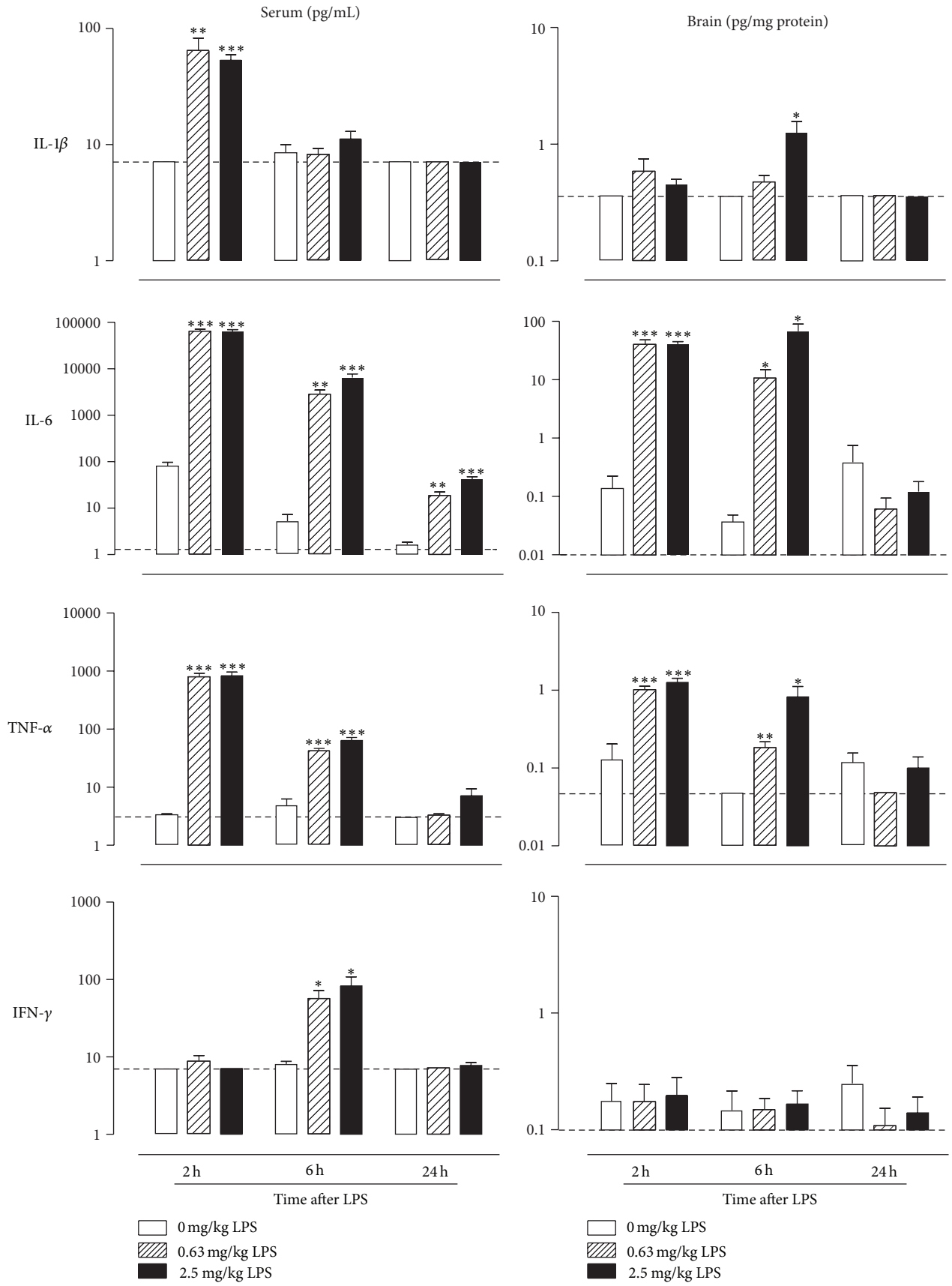
The stressed-induced hyperthermia paradigm reflects a physiological response to mild stress exposure and is sensitive to treatment with anxiolytic drugs [38]. The measure for anxiety in this paradigm is the increase in body temperature in response to the mild stress of measuring rectal temperature. rmANOVA revealed a significant interaction for stress × dose at all time points tested (2 h: $F(3, 36) = 5.4$, $P < 0.01$; 6 h: $F(3, 36) = 14.0$, $P < 0.001$; and 24 h: $F(3, 36) = 21.3$, $P < 0.001$). Post hoc analysis demonstrated that, at 2 h after LPS, there was a dose-dependent decrease in both Temp1 and Temp2, which was significant for Temp1 at 1.25 mg/kg and for Temp2 in all LPS-treated mice (0.31, 0.63, and 1.25 mg/kg LPS) (Figure 4, SIH). As LPS lowered both Temp1 and Temp2, there was no SIH effect in any of the LPS-treated mice, while it remained significant in control animals. At 6 h and 24 h following LPS, Temp1 was significantly increased in LPS-treated mice (0.31, 0.63, and 1.25 mg/kg), but there was no significant difference in Temp2 between LPS-challenged and control mice. At these time points, there was a significant SIH effect in all groups.

In the tail suspension test, behavioral despair was evaluated by measuring the time during which an animal remains immobile after being suspended by the tail. Factorial ANOVA revealed a trend for the factor dose at 6 h after LPS ($F(3, 35) = 2.3$, $P = 0.09$), but no statistical significance was found at 2 h or 24 h. Explorative post hoc analysis revealed that, at 6 h after LPS administration, mice treated with 0.63 mg/kg LPS, but not 0.31 or 1.25 mg/kg LPS-treated animals, showed a slightly increased immobility time (Figure 4, TST).

Behavioral despair in the forced swim paradigm was evaluated by measuring the time during which a rodent remains immobile after being placed in a water-filled cylinder from which it cannot escape. At 6 h after LPS, a trend was found for the factor dose ($F(3, 35) = 2.6$, $P = 0.07$), but no statistical significance was found for any of the other time points. Explorative post hoc analysis revealed that, at 6 h after administration, LPS induced a slight increase in immobility time that was significant in the 1.25, but not in the 0.31 or 0.63 mg/kg LPS-treated animals (Figure 4, FST). At 24 h after LPS injection, animals treated with 0.63 mg/kg remained immobile for a longer period than control animals. However, this increased immobility at 24 h after injection was not seen in mice exposed to 0.31 or 1.25 mg/kg LPS.

The sucrose preference test, in which the preference of an animal for a sweetened solution versus water is measured, is a commonly used rodent model to evaluate anhedonia. Our experiment consisted of two phases. The purpose of the *familiarization phase* was to assess normal daily intake volume, familiarize the animals with exposure to sucrose, and determine the effect of different sucrose concentrations on sucrose preference. rmANOVA revealed that, for total intake volume during the *familiarization phase,* there was a flavor × repeat × group interaction ($F(4, 45) = 5.8$, $P < 0.001$). Furthermore, a main effect of group ($F(4, 45) = 20.6$, $P < 0.001$) was found for sucrose preference. Post hoc analysis demonstrated that total intake volume in the *familiarization phase* increased significantly when animals were exposed to both sucrose and water (D2 and D4), but only when the animals were retested (D4) with a sucrose concentration of 5 or 10% (Figure 5, top left panel). The levels of sucrose preference correspond to these findings, as sucrose preference was significantly lower in mice exposed to 1% or 2% sucrose, but not in mice receiving 5% sucrose, when compared to mice exposed to 10% sucrose (Figure 5, bottom left panel).

In the *test phase*, the effect of i.p. LPS administration on total intake volume and sucrose preference was assessed over time. rmANOVA revealed that there was a strong time × group interaction for total volume intake ($F(8, 90) = 8.5$, $P < 0.001$, $\varepsilon = 0.86$). Post hoc analysis indicated that, in the first 24 h after administration (D8), LPS reduced the total intake volume to less than half of the normal daily water intake, suggesting suppression of drinking as a consequence of sickness (Figure 5, top right panel). On the second day after LPS administration (D9), the LPS-induced reduction in total volume intake was no longer present in mice exposed to 10%



(a)

Figure 3: Continued.

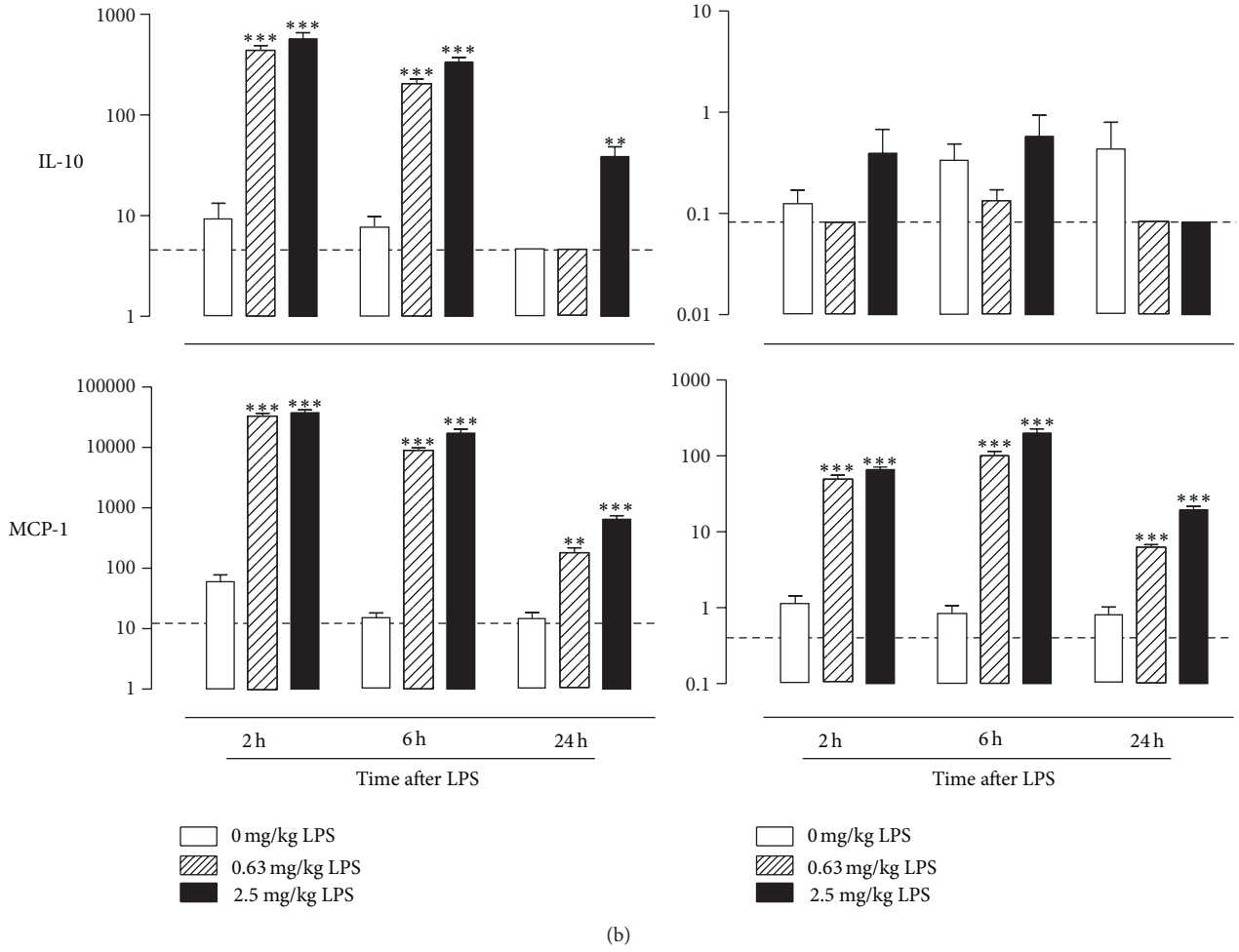


Figure 3: Peripheral LPS administration transiently increased cytokine levels in serum and brain. Comparison of selection of cytokines and one chemokine (MCP-1) in serum (left) and brain (right) after i.p. LPS administration. Dashed lines indicate the detection limit of measured cytokine. Note that serum concentrations are expressed in pg/mL, while brain levels are shown in pg/mg protein. Graphs are plotted as mean + SEM ($n = 12$ per group). Data were analyzed by ANOVA followed by independent samples t-test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to 0 mg/kg LPS.

sucrose solution, while it remained in the mice receiving 1, 2, or 5% sucrose. At D10, the total intake volume of all mice had returned to baseline levels.

For sucrose preference in the *test phase*, rmANOVA revealed a time × group interaction ($F(8, 90) = 4.3$, $P < 0.001$, $\varepsilon = 0.84$). In line with the total intake volume data, post hoc analysis demonstrated that, on D8, the sucrose preference was reduced in all LPS-treated animals (Figure 5, bottom right panel). In the following days, sucrose preference recovered depending on the sucrose concentration; as on D9, the sucrose preference for LPS-treated mice receiving 10% sucrose had returned to pre-LPS values, while for mice receiving 1, 2, or 5% sucrose this took up to D10.

4. Discussion

Based on the complexity and heterogeneity of depression, it is likely that several interacting systems underlie its pathogenesis. Findings from clinical studies indicate that inflammatory processes are associated with depression, at least in certain clinical subpopulations. For example, subsets of depressed patients show alterations of their peripheral immune system [4–7], and depression often occurs as a comorbidity in patients suffering from conditions characterized by a sustained, systemic inflammation [8–14]. Moreover, therapeutic stimulation of the immune system leads to depression in up to half of cancer and hepatitis C patients receiving proinflammatory cytokine treatment [15, 17].

Inflammation-associated depression is often studied in rodents by systemic administration of bacterial LPS, which is a potent activator of the immune system. Results from previous rodent studies indicate that systemic application of a single bolus of LPS leads to sickness behavior that peaks at 2–6 h, gradually fades over time, and is attenuated at 24–48 h after LPS injection (for a review, see Dantzer et al. 2008 [20]). There are some indications that depressive-like behavior can be separated from sickness 24 h after systemic LPS administration [22–24]. Contrastingly, other studies showed

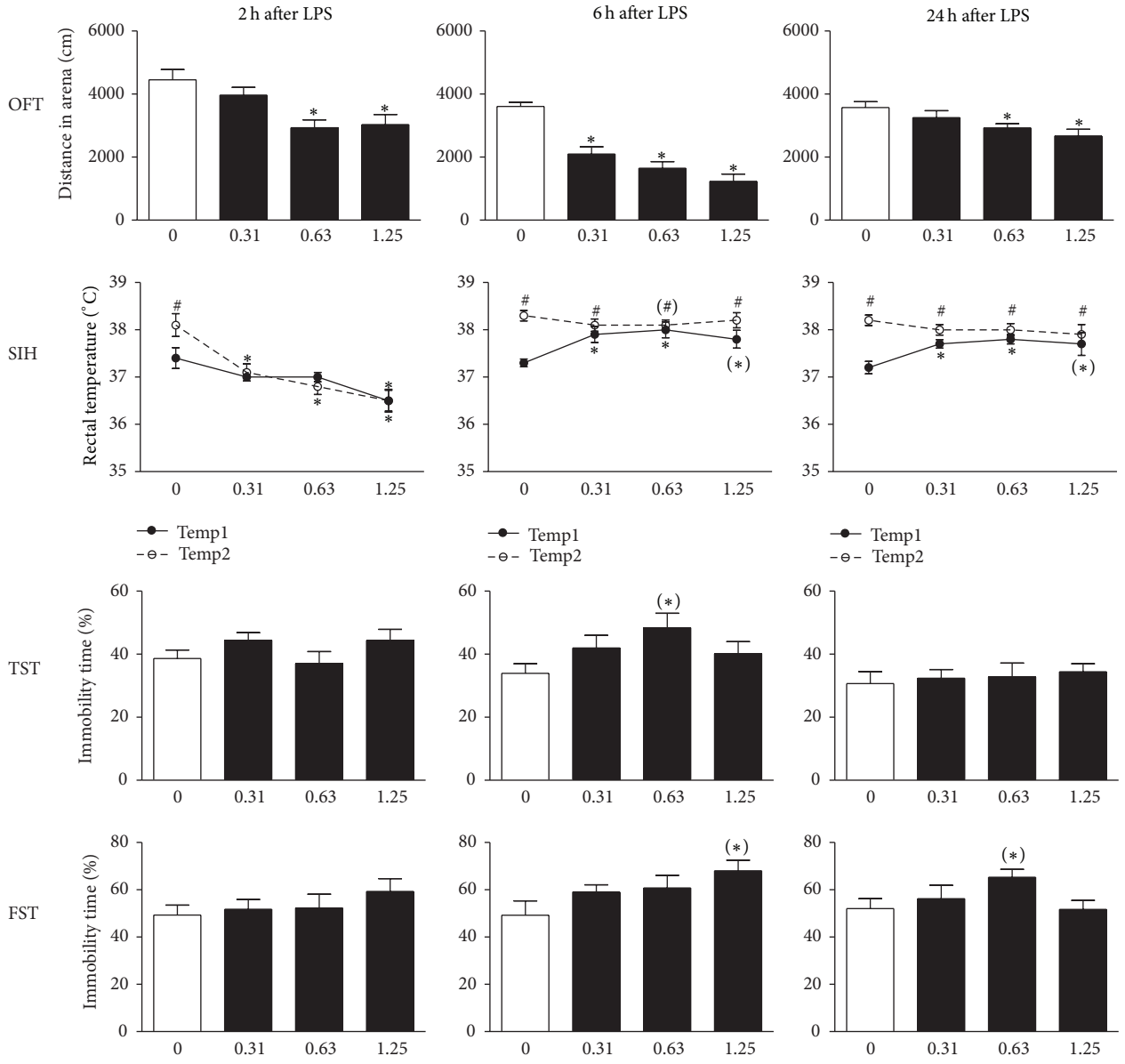


Figure 4: Intraperitoneal injection of LPS caused sickness, but no clear depressive-like behavior is observed. Peripheral immune activation caused a dose- and time-dependent reduction in locomotor activity in the open field test (OFT). However, a single i.p. injection of LPS did not induce clear anxiety or depressive-like behavior in the stress-induced hyperthermia (SIH) test, tail suspension test (TST), or forced swim test (FST). Graphs are plotted as mean + SEM ($n = 10$ per group). Data were analyzed by multivariate ANOVA followed by independent samples t-test. $^{*}P < 0.05$ compared to 0 mg/kg LPS group, $^{(*)}0.05 > P > 0.1$ compared to 0 mg/kg LPS group, $^{\#}P < 0.05$ compared to Temp1 (SIH) $^{(\#)}0.05 > P > 0.1$ compared to Temp1 (SIH).

that LPS-induced signs of sickness are still present at that time [27–30], making it difficult to compare results from different labs. Other factors complicating the interpretation of the existing literature include the difference in experimental design between studies and the use of only a single dose of LPS and/or a few time points. Consequently, assessing time- and dose-dependent changes in neuroinflammation and behavior following systemic LPS administration is not straightforward.

The present study was therefore designed to evaluate central effects of systemic LPS administration at several time points by combining multiple techniques to quantify neuroinflammation and behavioral changes. To our knowledge, such an extended and multidisciplinary approach has not yet been reported in this field. First, the kinetics of neuroinflammation following peripheral immune activation were assessed using a transgenic mouse line that carries the luciferase gene under the transcriptional control of the mouse GFAP promoter. GFAP is an intermediate filament protein that is predominantly expressed by astrocytes and is upregulated when astrocytes are activated [45]. This makes the bioluminescent GFAP-luc mouse model an ideal tool to

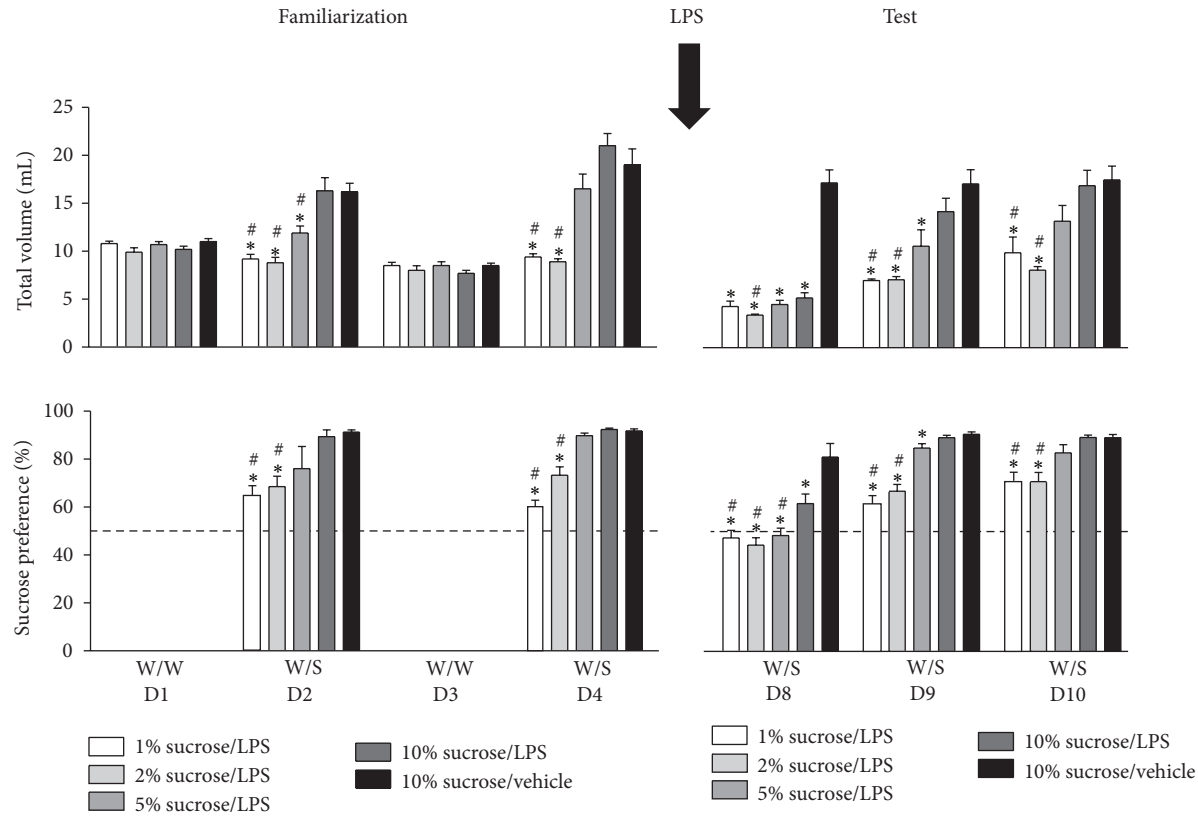


Figure 5: Intraperitoneal injection of LPS caused a transient reduction in total volume intake and sucrose preference in the sucrose preference test. During the familiarization phase of the experiment (left), animals were familiarized to the experimental setup. On day (D) 1 and D3, mice were exposed to 2 bottles of water (W/W), while on D2 and D4 one bottle contained water and the other bottle was filled with a 1, 2, 5, or 10% sucrose solution (W/S). Voluntary consumption of water or sucrose was measured during a period of 24 h for up to 3 days after systemic LPS administration (D8–D10). Dashed lines indicate chance level for sucrose preference. Graphs are plotted as mean + SEM ($n = 10$ per group). Data were analyzed by repeated measures ANOVA followed by independent samples t-test. $^{*}P < 0.05$ compared to 10% sucrose/vehicle group, $^{\#}P < 0.05$ compared to 10% sucrose/LPS group. W/W: water/water; W/S: water/sucrose.

quantify astrocyte activation, as marker of neuroinflammation, in living mice. Systemic LPS administration to these GFAP-luc mice led to a time- and dose-dependent increase in brain bioluminescence that peaked at 6 h after LPS administration and then gradually faded over time. The upregulation of GFAP at 6 h after systemic LPS injection demonstrates that astrocytes respond rapidly to a peripheral immune challenge. This early response of brain cells to peripheral immune activation has also been shown in another bioluminescent mouse model where systemic LPS administration induced a time- and dose-dependent increase in the expression of CCAAT enhancer binding protein (C/EBP), a regulator of inflammation [46]. However, C/EBP upregulation peaked at 24 h after LPS, a time point at which GFAP expression has decreased substantially, suggesting that astrocyte activation might be an early and short-lasting response to peripheral immune stimulation, while other inflammatory processes in the brain persist. GFAP-luc mice treated with the highest dose of LPS (2.5 mg/kg) died during scanning at 6 h after LPS. This was unexpected as the same dose was not lethal in NMRI mice tested throughout the rest of the study. One possible explanation for this discrepancy may be a strain-related difference in LPS sensitivity as previously described in models for acute lung injury [47] and inflammation-induced depression [48]. Other potential causes for the unexpected mortality in GFAP-luc mice treated with a high dose of LPS might be found in the experimental procedure to measure bioluminescence. It is possible, for example, that the toxic effects of isoflurane and/or potassium bound to luciferin become lethal in combination with a high dose of LPS.

To confirm glial activation using a different technique and another glial cell type, IBA1 expression was quantified in the hippocampal dentate gyrus of LPS-treated FVB wild-type mice. This brain structure is associated with stress and depression [40–42] and commonly studied in models of LPS-induced neuroinflammation [43, 44]. IBA1 is expressed in microglia, and its expression is elevated under pathological conditions [44, 49–51]. Consistent with astrocyte activation found in the GFAP-luc mouse, LPS-treated FVB wild-type mice showed a robust increase in IBA1 reactivity in the dentate gyrus. These results indicate that microglia, in addition to astrocytes, also show signs of activation in

response to systemic LPS administration and are in line with previous reports of increased IBA1 immunoreactivity in the hippocampus of LPS-treated mice [44, 52].

Acute systemic LPS administration is known to induce a transient release of cytokines in the periphery and CNS [37, 53, 54]. In agreement with the literature, the present study showed that serum levels of cytokines that are involved in the acute phase response of inflammation (IL-1β, IL-6, and TNF-α) were upregulated 2 h after peripheral LPS administration. Serum levels of IFN-γ, however, were only increased 6 h posttreatment, suggesting that the release of this cytokine was probably not triggered by LPS directly, but by downstream effects of earlier released cytokines. Serum levels of most proinflammatory cytokines had returned to baseline values at 24 h. However, at this time point, the serum levels of IL-6 and the chemokine MCP-1 were still slightly elevated in all LPS-treated mice, demonstrating that the immune system was still mildly activated in the periphery. IL-10, an anti-inflammatory cytokine that plays a role in regulating the intensity and duration of the inflammatory response, remained elevated in the serum of mice treated with a high dose of LPS. The fact that IL-10 levels were no longer elevated at 24 h in the serum of mice treated with a low LPS dose indicates that anti-inflammatory pathways return to baseline quicker after a less pronounced immune activation.

Cytokines from the periphery can pass the blood-brain barrier (BBB) and reach the brain through humoral, neural, and cellular pathways [55–57]. LPS has been shown to affect BBB permeability in several ways. Apart from early findings that LPS disrupts the BBB, LPS is now also known to exert direct effects on tight junction regulation [58] and cytokine release from endothelial cells in the brain [59]. However, the present study did not measure the integrity of the BBB and did not account for the fact that cytokines from the periphery can enter the brain through a leaky BBB. Despite this limitation, it was found that the time-dependent brain profiles of IL-6, TNF-α, and MCP-1 matched the serum profile, suggesting that these cytokines are expressed at a similar rate in the brain and/or that they can easily cross the BBB. Although IL-1β is known to pass the BBB [60], its brain levels were only significantly elevated at 6 h after LPS in mice receiving 2.5 mg/kg, but not in mice receiving 0.63 mg/kg LPS. These findings are in line with results described by Puentener and colleagues [54], who did not find an increase in IL-1β brain levels at 3 hours after acute i.p. administration. Erickson and Banks, in contrast, described an elevation of IL-1β brain levels in mice at 24 h after a single dose of LPS [37]. The present study was unable to reliably detect brain levels of IFN-γ and IL-10. Based on the large number of samples below detection limit in all treatment groups, this was likely due to a sensitivity issue and not to lack of cytokine levels in the brain. However, the strong increase in brain levels of IL-6, TNF-α, and MCP-1 confirmed that systemic LPS administration leads to a proinflammatory status in the brain. The brain levels of most cytokines returned to baseline at 24 h, while levels of the chemoattractant MCP-1 remained elevated. This indicates that there is still mild neuroinflammation present at this time point and coincides with the time course of astrocyte activation in the GFAP-luc mouse model and IBA1 immunoreactivity in the dentate gyrus of LPS-treated mice. This study did not account for regional differences of cytokine profiles in the brain. However, results from several other studies have pointed out that there might be a spatiotemporal component to LPS-induced cytokine production in the brain [53, 61–63]. Future research focusing on the identification of local changes in neuroinflammation may help to identify brain areas that are involved in inflammation-associated depression.

In addition to evaluating the LPS-induced peripheral and central immune responses as described previously, the second aim of this study was to investigate the main and side effects of peripheral LPS administration on behavior. Some indications already exist that, at 24 h after acute peripheral LPS injection, depressive-like behavior can be observed in rodents. However, the nature and characteristics of LPS-induced sickness behavior can substantially confound measurements of depressive-like behavior in commonly used paradigms. For example, sick animals show reduced motor activity which can confound measures of immobility, used to estimate despair in inescapable conditions (e.g., tail suspension and forced swim test) [20]. Therefore, studies focusing on depressive-like behavior should also include measures of sickness. Several groups have already used a combination of behavioral tests for that purpose. In some of these studies, a time window was identified in which sickness had dissipated while depressive-like behavior was still present. However, findings from different labs often vary. Some groups showed that LPS-treated mice display increased immobility in the tail suspension and forced swim test at 24 h, a time point at which motor activity in the open field test had returned to baseline [23, 24]. In contrast, other groups still observed reduced locomotor activity as an indication of sickness at this time after LPS administration [29, 30, 64]. Studies measuring sickness by evaluating social behavior are also not clear on the duration of LPS-induced sickness. Some groups, for example, have shown that social behavior returned to normal at 24 h after LPS [22, 29, 65], while others still report deficits in social behavior at this time point [27]. Hence, we evaluated the dose dependency and time course of LPS-induced changes in behavior across a panel of assays that are commonly used to study sickness, anxiety, and depressive-like behavior in rodents. Sickness, as measured by decreased locomotion in the OFT, occurred at 2 h after LPS treatment and had dissipated at 24 h in mice treated with a low dose of LPS. Animals treated with higher doses of LPS, however, still showed reduced locomotor activity at this point, indicating that sickness remained present in these mice. This timing coincided with signs of sickness seen in the SIH test where the baseline temperature (Temp1) of LPS-treated mice remained elevated at 24 h after LPS, thereby confounding measures of anxiety (dT). Depressive-like effects as evaluated by immobility time in the TST and FST were very low at all measured time points and can be considered biologically irrelevant here due to the cooccurrence of sickness. Furthermore, it is worth to note that we used naïve mice at each time point in our behavioral tests to avoid differences in confounding habituation effects (due to repeated testing) between sick and control animals.

From the sucrose preference experiment results, it becomes clear that the concentration of sucrose is a key factor for sucrose preference in mice. As seen on the last day of the *familiarization phase* (D4), the sucrose preference increased with sucrose concentration, with a ceiling effect being reached at 5% sucrose. Mice exposed to 5–10% sucrose also clearly drank much more than their normal daily intake, that is, on a day where they were exposed to water only. However, this was not the case in mice receiving 1-2% sucrose, showing that the total volume intake also depends on the sucrose concentration. Moreover, our data reveal that LPS reduced sucrose preference in a time-dependent manner. These findings are in line with previous results showing that LPS administration to mice decreased their sucrose consumption [23] and sucrose preference [22] for up to 2 days after administration, while leaving their water and food intake unaltered [23]. Despite the fact that, in our study, there was also no difference in water intake between treatment groups during the first 24 h after LPS administration (data not shown), it is important to mention that, at this time, the total volume intake in LPS-treated mice was reduced to approximately half of the normal daily intake. This suppressed drinking suggests that sickness still seems to be a confounding factor when measuring sucrose preference during the first 24 h after LPS administration and points out that caution is needed when interpreting LPS-induced reduction in sucrose preference as a measure of anhedonia.

Our data clearly show that acute systemic administration of LPS leads to a strong but ephemeral activation of the peripheral immune system with accompanying neuroinflammation and behavioral effects. Inflammation-associated depression in humans, however, is linked to chronic, persistent inflammation [21, 66]. This makes acute LPS administration to mice a less attractive translational model for inflammation-associated depression in humans. Interestingly, Kubera and coworkers recently described a mouse model in which repeated LPS injections given at one-month intervals induced a chronic state of anhedonia, indicating that chronic LPS administration might be a more relevant approach to induce depressive-like behavior [67]. In that study, the prolonged anhedonia in response to repeated LPS administration was only observed in female, but not in male mice. In another study, a less elaborate model of repeated LPS administration was shown to induce depressive-like behavior in absence of sickness in male rats [61]. It is possible that, as hypothesized for the human situation, a chronic inflammatory tone is needed to elicit depressive-like behavior in rodents. However, future work is needed to evaluate whether repeated LPS administration in rodents is a more valid model of inflammation-associated depression.

5. Conclusion

The present set of experiments using various assays and readouts confirmed that there is a strong crosstalk between the immune system and the brain, both on a neuroimmune and neurobehavioral level. Acute systemic LPS administration in mice caused a marked but transient increase in pro- and anti-inflammatory cytokines in the periphery. The time course of the systemic inflammation coincided with neuroinflammation as seen by astrocyte activation in GFAP-luc mouse, increased IBA1 immunoreactivity in the hippocampus, and elevated cytokine levels in the brain. Moreover, thorough investigation of several primary parameters across a panel of behavioral assays showed that systemic LPS administration induced sickness lasting for up to 48 hours. This time-dependent profile coincided with mild depressive-like behavior. However, due to overlapping time windows and rather mild effects on depressive-like behavior per se, it is not possible to separate sickness from depressive-like behavior in the present rodent model.

Conflict of Interests

The authors have no conflict of interests to declare.

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Review Article

Possible Involvement of TLRs and Hemichannels in Stress-Induced CNS Dysfunction via Mastocytes, and Glia Activation

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In the central nervous system (CNS), mastocytes and glial cells (microglia, astrocytes and oligodendrocytes) function as sensors of neuroinflammatory conditions, responding to stress triggers or becoming sensitized to subsequent proinflammatory challenges. The corticotropin-releasing hormone and glucocorticoids are critical players in stress-induced mastocyte degranulation and potentiation of glial inflammatory responses, respectively. Mastocytes and glial cells express different toll-like receptor (TLR) family members, and their activation via proinflammatory molecules can increase the expression of connexin hemichannels and pannexin channels in glial cells. These membrane pores are oligohexamers of the corresponding protein subunits located in the cell surface. They allow ATP release and Ca^{2+} influx, which are two important elements of inflammation. Consequently, activated microglia and astrocytes release ATP and glutamate, affecting myelinization, neuronal development, and survival. Binding of ligands to TLRs induces a cascade of intracellular events leading to activation of several transcription factors that regulate the expression of many genes involved in inflammation. During pregnancy, the previous responses promoted by viral infections and other proinflammatory conditions are common and might predispose the offspring to develop psychiatric disorders and neurological diseases. Such disorders could eventually be potentiated by stress and might be part of the etiopathogenesis of CNS dysfunctions including autism spectrum disorders and schizophrenia.

1. Introduction

Signaling between nervous and immune systems is in part due to the fact that these two systems share ligands and receptors. The cellular components involved in these interactions within the central nervous system (CNS) are mainly mastocytes, also called mast cells, and glia. In human brain, mastocytes are very scarce and are preferentially located in perivascular territories. By contrast, glial cells comprise about 90% of the total cell content in the CNS and are classified as microglia and macroglia (astrocytes, oligodendrocytes, and ependymal cells) [1]. Representative of the immune system in the CNS are mastocytes and microglia, two cell types derived from hematopoietic cells of the bone marrow that migrate to the brain before closure of the blood brain barrier (BBB) [2, 3].

The CNS challenged by different aggressions frequently elicits immune and inflammatory responses [4, 5]. Mastocytes and microglia are efficient sensors of adverse endogenous or exogenous conditions of the CNS [2, 6]. Moreover, stress conditions induce rapid mastocyte degranulation via the hypothalamic peptide corticotropin-releasing hormone (CRH) [7] and exogenous danger molecules like polyinosinic-polycytidylic acid (poly (I:C)), bacterial lipopolysaccharide (LPS), and peptidoglycan (PGN), which are detected by mastocytes and microglia via toll-like receptors (TLRs) [8, 9]. Also, glucocorticoids (GCs) play a relevant role in stress-induced potentiation of neuroinflammatory responses by sensitizing microglia to proinflammatory stimuli [10]. As part of these responses, glial TLRs, connexin hemichannels (Cx HCs), pannexin (Panx) channels might be key players in acute and chronic neurodegenerative diseases

characterized by open BBB, demyelinization, and neuronal degeneration [11].

The causes of various chronic diseases that affect the CNS, such as Alzheimer's disease (AD), Parkinson's disease (PD), and multiple sclerosis (MS), are complex and can be related to multiple factors. Notably, the innate host defense has been demonstrated to play an active role in promoting neurodegeneration [12, 13]. However, the possible role of these cellular and molecular elements during brain ontogenesis and the consequences in the adult CNS remain unknown. This review presents possible implications of glial toll-like receptors (TLRs) and Cx HC and Panx channels activation after potentiation by stress in CNS dysfunctions.

During pregnancy, viral infections are common and emerge to predispose the offspring to develop psychiatric diseases [14, 15]. Viral mimic polyinosinic:polycytidylic acid [poli (I:C)] resembles the structure of double-stranded RNA (dsRNA) generated in host cells during viral replication, and it is recognized by TLR3 that activates the innate immune response [16]. The administration of poly (I:C) is a way to trigger the innate immune response, which mimics the early phase of viral infections [17], and avoids the use of infectious agents, and treatments can be standardized and experiments may be easily compared [18]. All together, they represent an interesting area because perinatal infections, particularly those of viral etiology, are frequent and have been associated with diverse alterations of adult CNS, including schizophrenia and autism [19, 20].

2. Toll-Like Receptors (TLRs): Their Expression and Functions in Brain Cells

TLRs are highly conserved germ line-encoded pattern-recognition receptors that initiate innate immune responses via recognition of pathogen-associated molecular patterns (PAMPs) as well by recognition of danger-associated molecular patterns (DAMPS) that correspond to endogenous ligands released after tissue injury or cellular stress, such ATP, histones, heat-shock proteins, mRNA, high-mobility group box-1 protein (HMGB1), surfactant proteins A and D, and mitochondrial proteins [21]. Activation of TLRs triggers a cascade of intracellular events leading to activation of several transcription factors, including NF-κB, activator protein-1 (AP-1), and IFN-regulatory factor-3 (IRF-3) and -7 that regulate the expression of various cytokines and chemokines, responses that are performed in the CNS mainly by mastocytes and microglia. In addition, activation of innate immune responses via TLRs is a prerequisite for the generation of adaptive immune responses [22] that become relevant in autoimmune diseases such as experimental autoimmune encephalomyelitis (EAE).

The number of molecular members that comprise the TLR family is ten in humans (TLRs 1–10) and twelve in mice (TLRs 1–9; TLRs 11–13) [22]. Some TLRs can be expressed on the cell surface (TLRs 1, 2, 4, 5, 6, and 10) or in intracellular compartments (TLRs 3, 7/8, and 9), but others can be found in both the cell membrane and intracellular compartments (TLR3 and TLR7; endosomes and endoplasmic reticulum) [21]. Each TLR detects distinct PAMPs derived from viruses, bacteria, mycobacteria, fungi, or parasites. For example, TLR3 and TLR7/8 detect ds and single-stranded (ss) RNAs from virus, respectively; TLR4 responds to LPS from Gram-negative bacteria; and TLR9 senses bacterial DNA that contains unmethylated cytosine-guanosine dinucleotides (CpG) [22–25].

In the adult brain, mastocytes are mainly found in leptomeninges [2] and thalamus close to the BBB [26, 27], but they are also present early in brain ontogeny [28, 29]. Mastocytes can be activated by antigens that induce crosslinking of IgE bound to mast cells, CD47 recognition, calcium ionophore, ATP, compound 48/80, and also by recognition of DAMPS or PAMPS [26, 27]. If these activators bind to mastocytes for a short period of time (from seconds to a few minutes), they lead to rapid degranulation and release bioamines, proteoglycans, proteases, ATP, TNF-α and chemokines stored in preformed granules, whereas activations of longer durations lead to the release of newly formed cytokine (TNF-α, IL1β, and granulocyte macrophage colony-stimulating factor (GM-CSF)), and chemokine (C–C motif) ligand 3 (CCL3), enzymes (tryptase, chymase, carboxypeptidase), lipid mediators (prostaglandins, leukotrienes, thromboxanes, and platelet-activating factor), and nitric oxide (NO), mediating the recruitment of effector cells, fluid extravasation, and tissue inflammation [30, 31].

Murine mastocytes express the mRNA of TLRs 1–4 and 6–9 but not TLR5 [32–36]. Moreover, human mastocytes express the mRNA of TLRs 1–10 with the exception of TLR8 [9, 37–39]. In mastocytes, TLR ligands, such as poly (I:C), LPS, R-848, and CpG oligodeoxynucleotide, promote IL-6 and TNF-α secretion as well as regulated upon activation, normal T cell expressed and secreted (RANTES) and macrophage inflammatory protein (MIP) without significant degranulation [35, 38, 40, 41]. More specifically, in rodent mastocytes, binding of LPS to TLR4 induces the release of *de novo* expressed (without degranulation) and secreted TNF-α, IL-5, IL-10, and IL-13 but not GM-CSF, IL-1, or leukotriene C4 (LTC4), while binding of PGN to TLR2 induces degranulation that includes histamine release [9, 34, 37].

In three different mouse models, where TLR3, TLR4, and TLR7 were specifically deleted in mastocytes, the recruitment of effector $CD8^+$ T cells, neutrophils, and dendritic cells, respectively, was totally avoided after agonist stimulation [33, 42, 43]. This implies that mastocytes recognize, respond, and coordinate immune responses, features that are suppressed by TRLs 3, 4, and 7.

Not only ligands, but also immunological host environments are decisive for mastocyte activity. In human mastocytes, prolonged lymphotoxin-alpha (LTA) and PGN exposure downregulate FcεRI, decreasing degranulation products after an antigen crosslinking reaction [39]. Poly (I:C) treatment also decreases degranulation in an *in vitro* allergic model, affecting mastocyte adhesion to fibronectin and vitronectin through conformational inactivation of CD29, the receptor of fibronectin [44]. Moreover, LPS and PGN induce mastocytes migration *in vitro* after brief treatment with IL-6 and CCL5/RANTES, respectively [45].

The activation and migration of mastocytes occur in several neurologic disorders including MS [46, 47], PD [48], amyotrophic lateral sclerosis (ALS) [49, 50], AD [51], traumatic injury [52], ischemic and hemorrhagic stroke [53, 54], and viral infections [55]. Mastocytes activation and migration are critical for the increased BBB permeability and progression of neuroinflammation. Mastocytes also degranulate upon recognition of myelin basic protein and purinergic P2 receptors [56]. Additionally, proteases released during mastocyte degranulation can also degrade myelin components [57], contributing to myelin damage in the CNS and peripheral nervous system.

Microglia can rapidly respond to pathogens through their TLRs but do not sense apoptotic cells through the same mechanism [58, 59]. They express mRNAs encoding for TLRs 1 to 9. Moreover, levels of TLRs expressed by microglia vary depending on the stages of development or pathological conditions [8]. TLR activation induces a cascade of intracellular events leading to the activation of several transcription factors, including NF-κB, AP-1, IRF-3, and IRF-7 that regulate the expression of many molecular elements of inflammatory responses [60].

In human microglia, activation of TLR3 by agonists such as poly (I:C) induces a strong proinflammatory response that allows microglia to mediate the development of T-helper 1 (Th1) cells [61]. Moreover, infection with the West Nile virus (a retrovirus that produces dsRNA) in mice lacking TLR3 shows reduced microglial activation and more resistance to lethal infection with reduced viral load and inflammatory responses in the brain compared to wild-type mice [62].

Mastocytes release several cytokines in response to TLR2 activation including TNF-α, IL-4, IL-5, IL-6, and IL-13. Meanwhile, the activation of TLR4 causes release of TNF-α, IL-6, IL-13, IL-5, IL-10, and eotaxin [34, 63–65]. Also, numerous chemokines including CCL5/RANTES, can also induce a proinflammatory profile in microglia [37, 38, 59, 66]. IL-33 derived from microglia modulates the activation of P2 receptors on mastocytes inducing secretion of IL-6, IL-13, and monocyte chemoattractant protein-1 (MIP-1), which in turn can modulate the microglia activity [67]. Tryptase is the main protease secreted by human mastocytes. It is elevated in the CSF of patients with MS [68]. It induces microglia to secrete TNF-α, IL-6 [69], and ROS and activate in microglia proteinase-activated receptor-2 (PAR-2), a G protein-coupled receptors widely expressed in neurons, astrocytes, and microglia that are implicated in the pathogenesis of ischemia and neurodegeneration [70], because it induces widespread inflammation [71–73]. The activation of microglial PAR-2 also upregulates $P2X_4$ receptors and promotes release of brain-derived neurotrophic factor, TNF-α, and IL-6 that upregulate the expression mastocyte of PAR-2, which results in activation and release of TNF-α [67].

It is interesting to note that mastocytes but not microglia have been described to be the first responder in CNS injuries, such as perinatal hypoxia-ischemia. Many cells produce TNF-α in response to several stimuli, but mastocytes store TNF-α in granules, and thus they can release it before other cells including microglia and endothelial cells. Additionally, the recruitment and activation of mastocytes occur previous to responses elicited by neurons, glia, and endothelial cells. Therefore, mastocytes initiate acute inflammations in response to a stimulus, and when inhibited, the brain damage decreases, as observed when the early mastocyte response is inhibited with cromolyn (a mastocyte stabilizer), and then significant neuroprotection is observed [74].

A strong link between LPS, the TLR4 agonist, and brain injury both in fetal and newborn animals has been demonstrated [75]. LPS injected into developing mouse and rat brains has been shown to induce injury in white matter [76]. Moreover, systemic LPS administration to preterm fetal sheep induces cerebellar white matter injury [77], and *in vitro* assays demonstrate that TLR4 gene deletion prevents LPS-induced oligodendrocyte death [78].

In astrocytes, TLRs mediate the first step of innate immune cell activation. The expression of TLRs is limited in astrocytes, probably because of the neuroectodermal origin of astroglia [79]. These cells express TLR2, which increases in response to proinflammatory stimuli [22, 80]. They also express TLR3 that responds to poly (I:C), hence producing among other cytokines IL-6 that contributes to inflammation in humans and mice [80–82]. The gene profile of astrocytes activated via TLR3 shows neuroprotective mediators and cell growth factors, that is, differentiation and migration molecules comprising a neuroprotective response rather than a proinflammatory phenotype [83, 84].

TLR4 has been shown to participate in stroke-caused brain damage [85–87] and in AD [88, 89]. Likewise, TLR4 could play a pivotal role in demyelinating diseases, such as MS [90]. TLR activation in astrocytes also induces the release of several cytokines and chemokines [91]. Both TLR agonists and cytokines induce the expression of chemokines CCL2, CCL3, CCL5, intercellular cell adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1). Moreover, LPS and poly (I:C) induce the production of IL-6, TNF-α, IFN-α4, IFN-β, and iNOS [80]. In addition, poly (I:C) activation induces CXCL-10 production [92]. LPS and dsRNA in parallel induce astrocyte activation, which leads to IL-1α, IL-1β, IL-6, TNF-α, GM-CSF, LTβ, and TGF-β3 secretion, although macrophage migration inhibitory factor (MIF) secretion is inhibited. However, no effect has been found on anti-inflammatory cytokines such as IL-2, IL-3, IL-4, IL-5, IL-10, TGF-β1, TGF-β2, and TNF-β [11, 93].

Recently, in addition to TLR2, TLR3, and TLR4, TLR1, TLR5, TLR6, and TLR7/8 have been found in astrocytes, but their functional roles remain unknown [22, 84]. Therefore, the understanding of the detailed mechanisms of TLR signaling in astrocyte activation in CNS inflammatory conditions still needs further investigation.

The expression and function of TLRs in oligodendrocytes, unlike other glial cells, have been poorly studied. Only TLR2, -3, and -4 have been evaluated [94], being these receptors related to the regulation of inflammatory processes, gliosis, and remyelination after injury [95, 96]. Knockout mice for TLR2 and TLR4 exposed to spinal cord injuries show a lower remyelination capacity, and thus it is believed that these receptors would have a key role in the formation of myelin [84].

Astrocyte dysfunction triggers primary microglial activation, which induces demyelination [78, 97]. Furthermore, injection of LPS in the bone marrow induces a rapid oligodendrocyte loss, followed by an increase in oligodendrocyte number [98]. After acute demyelination induced by LPS, a more widespread distribution of oligodendrocyte precursor cells is triggered by the activation of microglia/macrophages, which is an event that accelerates remyelination [99, 100].

Rats treated with zymosan, a TLR2 agonist, show oligodendrocyte and axonal loss without regeneration [98]. In addition, rats treated with LPS, that is, a TLR4 agonist, show oligodendrocyte death and demyelination [76, 101]. Also, LPS-induced spinal cord damage shows significant demyelinization associated with an important reduction in the amount of oligodendrocytes [102]. Other researchers have shown that TNF-α and TNFR1 play a relevant role in oligodendrocyte death induced by TLR activation [103–105]. However, Bsibsi et al. [100] showed that zymosan and LPS reduce survival, differentiation, and myelin-like membrane formation, while poly (I:C) triggers apoptosis in rat oligodendrocyte cultures. These findings suggest that TLRs play a pivotal role in oligodendrocyte differentiation and myelination, both in physiological and pathological conditions. Compared to other cell types, TLRs play direct roles in regulating various aspects of oligodendrocyte's behavior. However, the apparent contradiction between the effects of LPS and zymosan on oligodendrocytes in different models has not been clarified. Future research could help to determine the functionality of TLR receptors in oligodendrocytes under physiological and pathological conditions.

With regard to the neuroendocrine modulation of the activity of TLRs, this can take local, regional, and systemic routes [106]. Local components include neuropeptides such as substance P, CRH, calcitonin gene-related peptide (CGRP), and endogenous opioids [106] released by peripheral nervous system. Among the regional components, the sympathetic and parasympathetic innervations release neurotransmitters (adrenaline and acetyl choline), and neuropeptides (neuropeptide Y or vasoactive intestinal peptide (VIP)) play a relevant role. Also at a regional level, a neuronal component regulates immunity through the innervation of immune organs and release of noradrenaline, and also a hormonal component regulates immunity systemically by means of adrenaline released from the medulla of the adrenal glands [106], whereas the systemic factors include the neuroendocrine system through the hypothalamic-pituitary-adrenal (HPA) axis and the anti-inflammatory effects of GCs. Furthermore, neuropeptides including cholecystokinin (CCK), somatostatin, melanocyte-stimulating hormone (MSH), VIP, and gastrin also reduce the inflammatory response [107].

Additionally, IL-1β participates in several aspects of the immune response to infections such as regulation of inflammation and modulation of adaptive immune responses against viral infections [108, 109]. The inflammasome is a multiprotein complex that activates a platform for caspase-1 and caspase-1-dependent proteolytic maturation and secretion of interleukin-1β (IL-1β). Several inflammasomes have been described being the NLRP3 inflammasome the most extensively studied [110]. It requires two signals. The signal 1 corresponds to TLR ligands or TNF-α, and the signal 2 includes ATP, amyloid-β (Aβ), K^+ efflux, pore-forming toxins, and silicic and uric acid crystals [111–113]. After TLR2 and TLR4 activation, secretion and maturation of cytokines IL-1β and IL-18 depend on caspase-1 cleavage of their premature forms. In both cases, inflammasome complex proteins mediate caspase-1 activation in the presence of high concentrations of extracellular ATP through activation of $P2X_7$ receptors [114, 115]. Activation of $P2X_7$ receptor leads to a large membrane pore formation identified as Panx1 channels [116, 117], which recently has been found critical for caspase-1 activation [116, 118]. Not only in immune cells but also in neurons and astrocytes, Panx1 recruitment mediates caspase-1 activation [119], suggesting that during infections, overall TLRs and Panx1 channels could enhance inflammatory responses.

3. Cx HCs and Panx1 Channels in Glial Cell and Mastocytes

One HC corresponds to one-half of a gap junction channel and is located at unapposed cell surfaces serving as communication pathway between the intra- and extracellular compartments. Two types of HCs are formed in most cells, and they are generally coexpressed [120]. One of them is formed by connexins (Cxs, 21 in humans) and the other by Panxs 1–3. HCs provide a membrane pathway for releasing signaling molecules (e.g., ATP, glutamate, PGE_2, and NAD^+) and thus are recognized as paracrine/autocrine communication pathways under normal and pathological conditions [121, 122]. Inflammation is a key condition in neurodegeneration that occurs in postischemic brain, diabetes, MS, PD, AD, and possibly in various other neurodegenerative diseases [123, 124]. In neuroinflammatory conditions, the successive activation of different glial cells via HCs has been partially demonstrated [125, 126], and mastocytes are likely to be involved in early steps of different pathological conditions (Figure 1).

As mentioned previously, the degranulation response of mastocytes is an early and rapid response and might require precise coordination where HCs could be essential. Mastocytes express Cxs 32 and 43 [127], but to our knowledge, it remains unknown whether they form functional HCs. In addition, no clear evidence of Panx1 expression in mastocytes has been published, but activation of $P2X_7$ receptors leads to the formation of membrane pores permeable to molecules up to about 900 kDa with single currents, similar to what has been described for Panx1 channels, along with histamine release [117, 128]. Since the degranulation process depends on influx of extracellular Ca^{2+} [129], it is possible that Panx1 channels participate in ATP release, and then ATP activates $P2X_7$ receptors, which are Ca^{2+} permeable, allowing the influx of Ca^{2+} required for the mastocyte degranulation response. Then, glial cells become involved and microglial cells respond before astrocytes (within several minutes to few hours).

In the normal CNS, microglial cells are in a resting state and are sparsely distributed. They express the macrophage

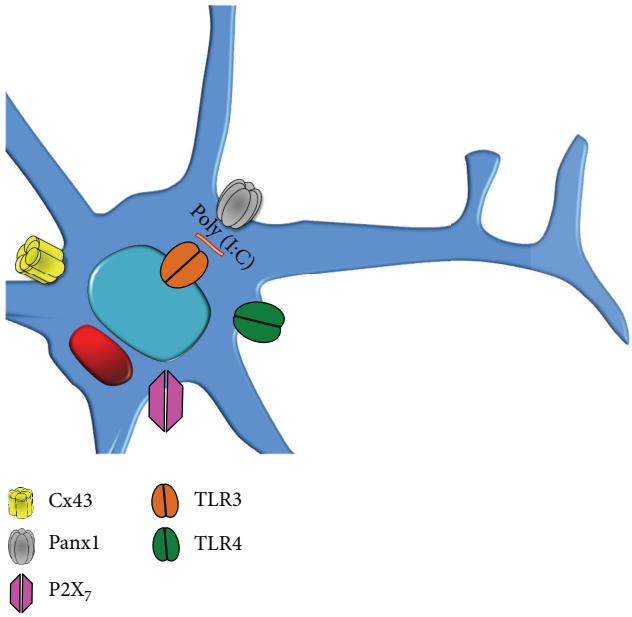

Figure 1: Expression of toll-like receptors in microglia and their relationship with pannexin channels and $P2X_7$ receptors. Microglia express TLRs 1 to 9, and in this figure only, TLRs 3 and 4 are shown responding to pathogen-associated molecular patterns of virus (TLR3) and Gram negative bacteria (TLR4). TLR3 is expressed in endosomal membranes (and also in cell surface) and recognizes nucleic acids of virus (dsRNA) and poly (I:C). We propose that microglia under activation with TLR ligands increase the expression of Panx1 channels and connexin HCs and the activity of $P2X_7$ receptors.

marker CD11b, low levels of CD45, and practically undetectable levels of major histocompatibility complex (MHC) class II molecules, CD40, and CD86. *In vitro*, the microglia activation process is characterized by an upregulation of CD45, MHC class II, and the costimulatory molecules CD40 and CD86 [130, 131]. The expression of MHC II antigens is a characteristic feature of antigen-presenting cells, and their coexpression with costimulatory molecules is a hallmark of microglial cells' ability to interact with other cells, such as T cells.

Activated, microglia proliferate and migrate to the injury site where they form cell aggregates and secrete pro- and anti-inflammatory cytokines and chemokines, NO, and growth factors [132]. The activation of microglia can be acute or chronic, and this would depend not only on the duration of an external stimulus but also on the quality of the stimulus (stress, infection, inflammation, and signals from damaged neurons) [133]. In fact, they show differences when activation is induced by stress or inflammation. For instance, acute stress induces morphological activation of microglia and increased c-Fos expression in the periaqueductal gray matter but not in the surrounding midbrain. If activation is chronic, it can lead to microglial overactivation followed by microglial degeneration [134]. Therefore, activated microglia secrete TNF-α and IL-1β, which in astrocytes induce opening of Cx43 HCs leading to the release of ATP and glutamate by astrocytes, which can kill neurons through the activation of Panx1 channels, $P2X_7$ receptors, and NMDA receptors in neurons [135].

Another way of cell-cell interaction used by activated microglia can be found in Cx- and Panx-based channels. Microglia express low to undetectable levels of Cx32, Cx36, Cx43, and Cx45 [136–139]. They also express Panx1, and treatment with $A\beta_{25-35}$ has been shown to increase its surface levels [126]. Similarly, the expression of Cx43 is upregulated in cultured rat/mouse microglia treated with LPS or TNF-α plus IFN-γ [136], calcium ionophore plus phorbol 12-myristate 13-acetate [140], or PGN derived from *Staphylococcus aureus* [139]. However, the possible functional role of Cx-based HCs expressed by activated microglia remains to be elucidated.

Under normal conditions, astrocytes are highly coupled with each other, forming intercellular networks [141], through which Ca^{2+} waves propagate [142]. Extracellular ATP acts as a paracrine messenger in these waves, since it activates purinergic receptors (P2X and P2Y) in astrocytes of surrounding cells, thus resulting in an increase of $[Ca^{2+}]_i$ [143]. The mechanisms for ATP release from astrocytes may include vesicle-mediated exocytosis [144] and diffusion through Cx43 HCs [125, 145, 146] and/or channels formed by Panx1 [147]. Astrocytes also release several transmitters called "gliotransmitters," including glutamate [148], GABA [149], ATP [150], and adenosine [151]. Increases in $[Ca^{2+}]_i$ can induce the release of gliotransmitters that promote increases in $[Ca^{2+}]_i$ in neighboring neurons, for example, through ATP- and glutamate receptor-dependent pathways [148]. The increased $[Ca^{2+}]_i$ occurs in local astroglia as well as in astrocytes located more distantly. Gliotransmitters might affect diverse neuronal functions including arborization and neuronal plasticity [142] as well as more complex functions such as fear memory [152]. Thus, astrocytic Cx HCs and Panx1 channels might be molecular targets to prevent undesired effects induced by stress.

Most astrocytes also express Cx30 and Cx43 [153], and at least Cx43 forms HCs that are activated by proinflammatory cytokines, hypoxia-reoxygenation, and high glucose [135]. For instance, LPS does not induce cell permeabilization to fluorescent dyes in primary cultures highly enriched with astrocytes of newborn brains, but astrocytes cocultured with microglia respond to LPS with a large increase in Cx43 HC activity [154]. Moreover, the effect of LPS is mimicked by exogenous applied TNF-α and IL-β, indicating that astrocytes do not respond to LPS in the absence of microglia. Moreover, astrocytes previously exposed for 24 h to medium conditioned by Aβ-treated microglia (CM-Aβ) are permeabilized via Cx43 HCs [126]. As part of the mechanism, TNF-α and IL-1β have been shown to mimic the effect of CM-Aβ, and neutralizing TNF-α with soluble receptors and IL-1β antagonists abrogated this effect [125]. Recent *in vivo* studies have demonstrated that Cx43 HCs are critical mediators of postischemic white and gray matter dysfunction and injury [155]. Moreover, upregulation of astroglial Panx1 channels and Cx43 HCs has been found using an experimental model of brain abscess [156], suggesting that both channel types could play an orchestrated function in some inflammatory responses. Cx43 HCs of reactive astrocytes favor the release of

excitotoxic compounds, ATP, and glutamate, which activate neuronal $P2X_7$ receptors, NMDA receptors, and Panx1 channels, hence promoting neurodegeneration [125]. Activation of neuronal Panx1 channels by ATP and glutamate released through Cx43 HCs from astrocytes exposed to CM-Aβ was shown to induce neuronal death [126]. Therefore, it has been proposed that blockade of astroglia and/or neuronal Cx HCs and Panx1 channels of the inflamed nervous system may represent a strategy to reduce neuronal loss in various pathological states [157–159]. Additionally, the effect of the maternal environment on the developing CNS in the offspring has been analyzed in fetal nonhuman primates. To this end, mothers were subject to a high-fat diet (HFD), and the CNS of the fetuses showed increased levels of IL-1β and IL-1 type 1 receptor, as well as a rise in microglia activation markers, suggesting the activation of the local inflammatory response [160]. Under the previous conditions, it is possible that microglia and astrocytes also present upregulation of HC activity, but this needs experimental demonstration.

Oligodendrocytes might respond within the same time frame as astrocytes, since they can communicate via gap junctions as previously described herein. These cells are responsible for producing and maintaining myelin from the earliest stages of embryonic development to adulthood [161]. Like other cells of the CNS, oligodendrocytes have low renewal capacity [162]. However, oligodendrocyte precursor cells induce remyelination, following the loss of myelin as a consequence of an injury [163]. Many of their functions are accomplished by the expression of a variety of interactions between Cx- and pannexin-based channels. Oligodendrocytes form gap junction channels with cell bodies of adjacent oligodendrocytes and between layers of myelin, called reflective gap junctions [164]; oligodendrocytes form gap junctions with astrocytes as well [165]. Collectively, this gap junction communicated network helps to absorb and remove extracellular K^+ and glutamate released during neuronal activity, thus generating a spatial buffer where ions and molecules are diluted among cell communicated via gap junction channels [165–167].

The study of demyelinating diseases, consisting of loss or destruction of myelin, has revealed Panx1 channels, Cx HCs, and gap junction channels as key factors in oligodendrocyte survival, as well as neuroprotection and myelin maintenance [168]. Oligodendrocytes express three different connexins: Cx29, Cx32, and Cx47 [169]. Cx32, but not Cx29 or Cx47, is known to form functional HCs. Moreover, by means of the qPCR technique, the mRNA of Panxs 1 and 2 was detected in primary cultures of oligodendrocytes obtained from optic nerves of 12-day-old rats. Both were located in somas as well as in the layers of the myelin sheath [170]. Extracellular ATP mediates the ischemic damage to oligodendrocytes and is partially explained by the activation of Panx1 channels [170].

Both genetic and/or inflammatory diseases triggered by viral or toxic sources may affect myelin formation (hypomyelinatin diseases) or its maintenance (demyelinating diseases) as it has been found in human diseases associated with HCs formed by mutated Cxs [161]. The first event in pathological manifestations of demyelinating disease of the CNS is the disruption of the BBB that leads to access of demyelinating antibodies [161, 171–174]. Also, activated T cells entering the CNS mediate the release of inflammatory cells, which together with activated microglia release proinflammatory cytokines that promote oligodendrocyte death *in vitro* [175–178]. TNF-α binding to its receptor can induce oligodendrocyte apoptosis directly [179]. Indirectly, TNF-α and IFN-γ can activate microglia and/or macrophage that destroy oligodendrocytes by oxidative stress [180, 181].

Myelin repair occurs after acute inflammatory lesions, such as MS. This repair is called remyelination, and its process, mediated by oligodendrocyte progenitor cells, is associated with functional recovery [163]. It has been shown that chemokine- (CXCL-) 2 and proinflammatory cytokines, such as IL-1β and IL-6, promote oligodendrocyte progenitor cell proliferation, differentiation, and remyelination [163]. Under inflammatory conditions, oligodendrocytes show upregulation of MHC I molecules, which are constitutively expressed, as well as Fas, IFN-γ, and TNF-α receptors (TNFRI-II), transforming them into targets for $CD8^+$ cells [175, 176, 182–185]. Under control conditions there is no expression of MHC II molecules in these cells [186, 187]. However, cultured oligodendrocytes treated with IFN-γ in the presence of the synthetic GC (dexamethasone) express MHC II molecules [188], suggesting that under stress they could interact with CD4 T lymphocytes and either activate immune reactions or become the targets of T-cell-mediated cytotoxic attack.

An excess of extracellular ATP is an activator of both innate and acquired immunities, acting as a DAMP that is chemotactic factor for neutrophils, and a strong regulator of activation, death, and survival of microglial cells [189–191]. Pathway for ATP release is highly variable and includes connexin HCs, Panx1 channels, volume-regulated anion channel (VRAC), purinergic $P2X_7$ receptor, and/or vesicular exocytosis [192–195]. Moreover, mastocytes represent an abundant source of ATP stored in granules that are released under activation conditions [196–198] such as specific (e.g., IgE + antigen) and nonspecific stimulation (e.g., stress, mechanic stimulation, and osmotic swelling). With regard to the participation of mastocytes in CNS alterations, ATP can be released by trauma-induced degranulation and thus stimulates adjacent neurites via P2X and P2Y receptors. Additionally, the neuropeptide SP released from nerve terminals upon bradykinin stimulation participates in nerve mastocyte communication [199]. This enables interactions between nerve and mast cells and initiates and represents the development of neuroimmunological synapses. Also, glial cells are involved in neuroimmune cross-communication, and ATP induces glial cells to release IL-1β, TNF-α, and IL-33. Therefore, ATP released from mastocytes is an important autocrine/paracrine/exocrine factor that mediates cross-communication between different cell types [200]. Moreover, human LAD2 mast cells stimulated with IgE, anti-IgE, or substance P (SP) secrete mitochondrial particles, mitochondrial DNA (mtDNA), and ATP in absence of cell death. Furthermore, mitochondria added to mast cells trigger degranulation and release of histamine, PGD_2, IL-8, TNF-α, and IL-1β, and this response is partially inhibited by DNAse and ATP receptor antagonists [201].

4. Activation of Glial Cells and Mastocytes during Stress and Infection

Only 30 min of immobilization stress can stimulate the HPA axis and cause degranulation in ~70% of rat dura mastocytes [202]. This response could be triggered by neurotensin (NT) and CRH acting on mastocytes increasing the permeability of the BBB [203–206]. As mentioned previously, activated mastocytes release proinflammatory cytokines and ATP among other bioactive compounds that promote microglia, and astrocyte activation and both reactive glia promote neuronal damage [123, 124]. Related to this, acute or chronic stress through GCs sensitizes microglia to a subsequent proinflammatory challenge [10], suggesting that stress should worsen the outcome of neuroinflammation. To our knowledge, it remains unknown if signal transduction of proinflammatory agents via TLRs and activity of HCs is enhanced by GCs or stress.

Related to the issue presented previously, various neurodegenerative disorders present activation of microglia in different brain regions [124] and restraint combined with water immersion induces massive microglial activation in the hippocampus, hypothalamus, thalamus, and periaqueductal gray matter [207, 208]. Although the precise mechanism of microglia activation induced by stress remains unknown, it is likely that bioactive molecules released by activated mastocytes (see what is mentioned previously) lead to the activation of microglia and, therefore, induce progression of neurodegenerative changes. In an *ex vivo* approach, rats were first pretreated *in vivo* with RU486 (GC receptor antagonist) and then exposed to an acute stressor (inescapable tail shock; IS), and 24 h later, hippocampal microglia were isolated and stimulated with LPS. Microglia obtained from rats not treated with a GC receptor antagonist showed an increase in gene expression of proinflammatory cytokines (IL-1β and IL-6). However, in rats pretreated with RU486, the sensitization of microglial to proinflammatory stimuli did not occur [10]. Astrocytic signaling is potentiated by GCs (i.e., methylprednisolone and dexamethasone) via long-range calcium waves, and an increase is observed in resting cytosolic Ca^{2+} levels, as well as the extent and amplitude of calcium wave propagation (twofold) compared to control conditions [209]. Furthermore, it is known that stress affects microglial function and viability during adulthood and early postnatal life [210]. Experiments both *in vitro* and *in vivo* have shown that stress hormones can affect the function and viability of microglia. However, little is known if stress during pregnancy affects microglia of the offspring. In a recent report, prenatal stress effects on microglia of the offspring were studied. In this model, prenatal stress during embryonic days 10–20 consisted of 20 min of forced swimming. In the offspring, a reduction in the number of immature microglia in the two main brain reservoirs of amoeboid microglia, corpus callosum, and internal capsule was observed. Moreover, accelerated microglial differentiation into ramified forms in the internal capsule and brain regions, such as the entorhinal cortex, parietal lobe neocortex, thalamus, and septum, was seen in the neonates in relation to an increase in plasma corticosterone in the pregnant dam [211].

The stimulation of microglial TLR3 with its ligand leads to the release of IL-6, IL-12, TNF-α, and IFN-γ among others (Figure 1). In connection to this, the importance of TLR in various CNS diseases (i.e., infection, trauma, stroke, neurodegeneration, and autoimmunity) has been described [212]. This is how viral infections have been implicated in the onset of MS by stimulation of TLR3 [105]. Additionally, in an animal model of schizophrenia, the stimulation of pregnant mothers with poly (I:C) results in reduced neuronal arborization of the offspring, which is correlated with a status of higher activation [213]. Interestingly, Cx HCs participate in neurite outgrowth [214] and release of ATP and glutamate [125], which also affect neuronal arborization [214, 215].

It is interesting to note that sensitivity to drug abuse behavior, as well the neuroinflammatory response to a subsequent proinflammatory challenge (as noted previously), is associated with stress and stress-induced release of GCs. Neuroinflammatory mediators derived from glia have an important role in the development of drug abuse [216]. This is how neuroinflammatory mediators, such as proinflammatory cytokines, are induced by opioids, psychostimulants, and alcohol, all of which modulate many effects including drug reward, dependence, tolerance, and analgesic properties. An interesting aspect is that drugs of abuse may directly activate microglial and astroglial cells via TLRs, which mediate the innate immune response to pathogens [216]. A key aspect is the timing of stress exposure relative to inflammatory challenge, and if a proinflammatory stimulus (e.g., LPS) is added immediately before stress exposure, stress induces an anti-inflammatory effect, which is reflected in the inhibition of the increase in brain IL-1β levels [217].

The importance of stress associated with infections is given by the fact that the acute or chronic stress sensitizes the inflammatory responses of the CNS to immunological challenges. Microglia show an increase in expression of MHC II, TLR4, and the F4/80 antigens. Therefore, stress changes the microenvironment of the CNS to a phenotype with inflammatory characteristics. One explanation to this phenomenon is that GCs sensitize microglia to infections [10, 218]. In peripheral blood monocytes from individuals under chronic stress, an increase in the expression of genes with promoter response elements for NF-κB is observed as well as allows expression of genes that have promoter elements for GC receptors [219]. Otherwise, in older stressed or chronically depressed adults, an increase in inflammatory response occurs when they are challenged with antigens, showing depressive characteristics and elevated levels of IL-6 after immunization with influenza vaccines. Further evidence that supports this notion comes from observations in older caregivers of patients with dementia, who also presented an elevation of IL-6 for over four weeks after vaccination with influenza vaccines, whereas this elevation was not observed in non stressed individuals [220].

Furthermore, stress worsens immunity and brain inflammation, which is important in MS and neuropsychiatric disorders [221–226]. Under stress, the neuropeptides CRH and NT are secreted and thus can activate microglia and mast cells, which in turn release molecules with proinflammatory properties. This results in maturation and activation of Th17

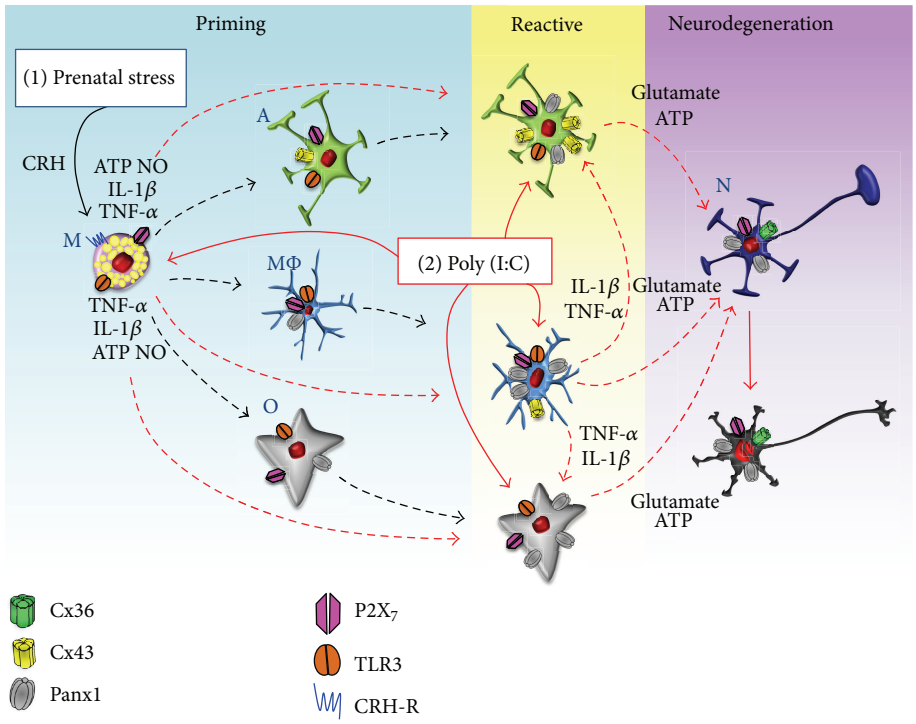

Figure 2: Model of the involvement of mastocytes and microglia in neuroinflammatory responses and potentiation of their responses by stress. Stress increases the levels of CRH and glucocorticoids which are critical players in stress-induced mastocytes (M) degranulation and potentiation of glial inflammatory responses (sensibilization). Furthermore, perinatal infections, particularly those of viral etiology (poly (I:C)), are frequent and have been associated with diverse alterations of CNS. Mastocytes and microglia (MΦ) express toll-like receptor 3 (TLR3). Activated microglia and mastocytes increase the hemichannel activity in reactive astrocytes (A) and oligodendrocytes (O). Both activated microglia and astrocytes release ATP and glutamate that induce neurodegeneration through the activation of $P2X_7$ receptors and Panx1 channels in neurons (N) (neurodegeneration) (modified from Orellana et al., 2011) [125].

autoimmune cells and disruption of the BBB that leads to T cells entry into the CNS enhancing the brain inflammation, which might support the pathogenesis of MS. NT also stimulates secretion of vascular endothelial growth factor (VEGF) and induces expression of CRH receptor-1 in mast cells [20, 206, 227]. Several lines of evidence associate microglia with the pathogenesis of MS because activation of microglia is prominent and precedes T-lymphocyte infiltration and demyelination [228]. Activated microglia release glutamate and NO causing neuronal death and BBB disruption [228, 229]. With regard to the participation of mastocytes in the pathogenesis of MS, patients with this disease show elevated levels of tryptase (that activate microglia) and histamine in cerebrospinal fluid (CSF) [68, 230]. Therefore, several lines of evidence suggest an important role of mastocytes and microglia in neuroinflammatory diseases [67]. Therefore, both cell types represent therapeutic targets to be considered for treatment of MS and other neuroinflammatory diseases.

Among the factors relevant to the development of autism spectrum disorders (ASD), stress during pregnancy and the first 6 months of postnatal life has been associated with increased risk of ASD [231]. Stress induces the secretion of CRH from the hypothalamus and activates the HPA axis [232]. As mentioned previously, CRH also activates mast cells, resulting in the release of several proinflammatory cytokines [233] including IL-6, which in turns may increase the BBB permeability [222, 234, 235].

Recently, a decrease in the mitochondrial function in approximately 60% of patients with autism has been demonstrated [236–238]. The brain of these patients shows lines of evidence of neuroinflammation [239–242], with high levels of mitochondrial DNA [243]. Additionally, elevated levels of NT that could activate mast cells have been detected in children with autism [244]. The involvement of mast cells and brain inflammation is related to mitochondrial fission and translocation to the cell surface during degranulation [245], which leads to release of ATP and mitochondrial DNA [243]. The importance of ATP is that it can maintain inflammation by activating mast cells [225, 246].

5. Concluding Remarks

Stress potentiates neuroinflammatory responses by sensitizing microglia to proinflammatory stimuli [10]. This is how prenatal stress modifies the phenotype, distribution, and activation statuses of microglia in the offspring [211]. Different stressors, together with the activation of the inflammatory immune response, enhance the effects of proinflammatory molecules or conditions, showing synergistic effects [247]. Viral infections are the most common causes of infection during prenatal life, and maternal respiratory infection can also increase the risk of the offspring to develop certain mental disorders. The most direct evidence for this comes from a prospective study of pregnant women

with medically documented respiratory infections, where the risk for schizophrenia in the offspring is increased 3-fold by infection in the second trimester [248]. Evidence that supports this phenomenon comes from models of cocultures between astroglia and microglia treated with dexamethasone. In these experiments, functional membrane properties of astrocytes in cocultures are differentially regulated, which might reflect steroid effects in adjacent glial components *in vivo*. In cocultures with 30% microglia, dexamethasone-treated cocultures show significant increased gap junctional intercellular communication [249], which could facilitate the propagation of inflammatory signal along astrocytic networks. Therefore, if a stressor is sufficiently sustained, this may reflect neurochemical processes that can make the organism more vulnerable to pathological stimuli producing behavioral and neurochemical responses [250, 251]. This can be reflected in an increased susceptibility to diseases of the nervous system, such as the progression of depressive disorders and anxiety, and can even affect the course of neurological diseases [250, 251]. Furthermore, activated microglia affect the expression of Cx HCs in astrocytes, which in turn increases the astrocytic ATP and glutamate release with deleterious consequences on neurons [125]. Therefore, these lines of evidence represent an aspect to be addressed in a model of stress in pregnant animals, in which one can analyze the effects of stress on microglia of the offspring in terms of activation and its effect on astrocytes, which could promote neuronal damage, with Cx HCs and Panx1 channels being possible therapeutic targets. Additionally, the synergistic effect of stress and stimulation with viral infection (for which RNA viral mimics poly (I:C)) has not been studied in offspring of pregnant females subjected to stress, which is also a novel approach and can be correlated with a possible susceptibility of offspring to diseases of the nervous system.

An important aspect is that when microglia are strongly activated, they remain in a preactivate state for years, which means that microglia are excessively responsive to even slight stimuli. This fact also has been linked to the activation of microglia by viral infections early in life and that can be later reactivated more rapidly compared to microglia in normal state [252, 253]. Therefore, the possibility of having microglia (using minocycline) and mastocytes activation (with GRH-R antagonists) as therapeutic targets opens the possibility of their modulation as treatment for various neuropsychiatric disorders, viral infections, and other neuroinflammatory pathologies of the CNS.

In summary, parental stress is proposed to induce potentiation of neuroinflammatory responses by first: activating directly mast cells through CRH recognition. Second: mast cells proinflammatory mediators prime microglia, astrocytes and olygodendrocytes, modifying their phenotype, distribution, and activation statuses in the offspring, but mainly promoting HC expression. Third: sensitized microglia exposed to inflammatory stimuli (i.e., TLR3 ligands) (Figure 1) are activated and secrete cytokines (TNF-α, IL-1β). They also show increased functional expression of Panx1 channels and Cx HCs through which ATP and glutamate are released to the extracellular milieu. Astrocyte and oligodendrocyte become activated and release ATP and glutamate in an HC depending way, and thus they promote neurodegeneration (Figure 2). Therefore, HCs represent a novel target with clinical applications in neuroinflammatory diseases.

Acknowledgments

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Review Article

Cellular and Molecular Mediators of Neuroinflammation in the Pathogenesis of Parkinson's Disease

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Neuroinflammation is a host-defense mechanism associated with restoration of normal structure and function of the brain and neutralization of an insult. Increasing neuropathological and biochemical evidence from the brains of individuals with Parkinson's disease (PD) provides strong evidence for activation of neuroinflammatory pathways. Microglia, the resident innate immune cells, may play a major role in the inflammatory process of the diseased brain of patients with PD. Although microglia forms the first line of defense for the neural parenchyma, uncontrolled activation of microglia may directly affect neurons by releasing various molecular mediators such as inflammatory cytokines (tumor necrosis factor-α, interleukin [IL]-6, and IL-1β), nitric oxide, prostaglandin E2, and reactive oxygen and nitrogen species. Moreover, recent studies have reported that activated microglia phagocytose not only damaged cell debris but also intact neighboring cells. This phenomenon further supports their active participation in self-enduring neuronal damage cycles. As the relationship between PD and neuroinflammation is being studied, there is a realization that both cellular and molecular mediators are most likely assisting pathological processes leading to disease progression. Here, we discuss mediators of neuroinflammation, which are known activators released from damaged parenchyma of the brain and result in neuronal degeneration in patients with PD.

1. Introduction

Parkinson's disease (PD) is a frequent neurological disorder of the basal ganglia characterized by the progressive loss of dopaminergic neurons, mainly in the substantia nigra pars compacta (SNpc) [1], cytoplasmic inclusions of aggregated proteins, and neuroinflammation [2, 3]. Several hypotheses have been postulated regarding the possible causes for neuronal degeneration in patients with PD. These include genetic factors, environmental toxins, mitochondrial dysfunction, and free radical-mediated cell death [4–6]. Although there is less evidence suggesting that neuroinflammation is the primary trigger causing neurodegeneration, preclinical and epidemiological data now strongly suggest that chronic neuroinflammation may be a slow and steady reason for neuronal dysfunction during the asymptomatic stage of PD [7]. Neuroinflammation induced by exposure to either infectious agents or toxicants with proinflammatory characteristics is now increasingly recognized as a major contributor to the pathogenesis of PD [8]. Whitton in 1988 initially suggested the involvement of inflammation in PD by describing upregulation of major histocompatibility complex (MHC) molecules in patients with PD [9]. The hallmarks of neuroinflammation are the presence of activated microglia and reactive astrocytes in the parenchyma (neurons, astrocytes, and endothelial cells) of the central nervous system (CNS), direct participation of the adaptive immune system, increased production of cytokines, chemokines, prostaglandins, a cascade of complement proteins, and reactive oxygen and nitrogen species (ROS/RNS), which in some cases can result in disruption of the blood-brain barrier (BBB) [10]. The extent to which neuroinflammation and peripheral immune responses contribute towards the development of PD or modify its course is not exactly known. In fact, dysregulation of the neuroimmune system has been postulated by many to be one of the underlying causes of the chronic nature of PD.

Several lines of evidence support the hypothesis that glial reactive and inflammatory processes participate in the

cascade of events leading to neuronal degeneration [11]. One of the earlier studies reporting neuroinflammation in PD involved a quantitative confirmation of the astroglial reaction using glial fibrillary acidic protein (GFAP) immunostaining in the substantia nigra (SN) of patients with PD [12]. Fundamental work by McGeer et al. [13], over two decades ago, first identified significantly increased levels of human leucocyte antigen-DR-positive microglia in the postmortem brains of patients with PD [14]. Following these reports, an increased number of activated microglial cells had consistently been reported in the neuroinflammatory pathogenesis of PD [11]. Initially, the pathological role played by these glial cells was not fully understood, but activated microglial cells have a deleterious effect on dopaminergic neurons. Microglial cells represent resident brain macrophages that are transformed into activated immunocompetent antigen-presenting cells during the pathological process [15]. Microglia in PD have been observed to grow densely in the striatum and SN with increased expression of proinflammatory mediators, including tumor necrosis factor alpha (TNF-α), interleukin-1β (IL-1β) [16], IL-2, IL-4, IL-6, transforming growth factor-α (TGF-α), cyclooxygenase-2 (COX-2), and inducible nitric oxide synthase (iNOS) [14]. TGF-β1 and β2 have also been detected by several investigators in the cerebrospinal fluid (CSF) and brain parenchyma of patients suffering from PD [17]. Taken together, these data indicate that glial cells are one of the most important cells involved in mediating neuroinflammatory events in PD [18].

In addition to glial cells, other cells may also participate in the neuroinflammatory processes in PD. Increasing evidence now demonstrates the involvement of both innate and adaptive immune responses in the pathophysiology of PD [19–21]. Innate immunity does not require the presence of a specific antigen to elicit an immune response, whereas adaptive immunity is activated when specific antigens are presented and recognized by lymphocytes. In contrast, endogenous pathological antigens that normally do not occur in physiological conditions may also initiate adaptive immune responses [22]. Indeed, Hirsch et al., in their various experiments, reported a small number of CD8-positive T lymphocytes in the vicinity of degenerating neurons in the SN of a patient with PD [18]. In line with this observation, an increased density of interferon-(IFN-) γ-positive cells with lymphocytes in the SN of patients with PD has also been reported [18]. Taken together, these data indicate that injured dopaminergic neurons release immunogenic mediators which have the potential to provoke detrimental innate and adaptive immune responses thereby amplifying the neuroinflammatory process in PD. Furthermore, mounting evidence suggests that BBB permeability may be modulated under neuroinflammatory conditions, and trafficking of leukocytes and peripheral macrophages into the brain becomes a normal process that must be tightly regulated to promote brain homeostasis and avoid the neuronal demise [10, 23]. These pathomechanisms not only produce complex crosstalk between the peripheral immune system and CNS but also highlight the interactions between microglial cells and other brain parenchymal cells [18]. Therefore, identifying and understanding the nature and role of the neuroinflammatory mediators involved in the pathogenesis of PD might provide us with various options to target these neuroinflammatory pathways to help curb neuronal death in PD. This review describes various cellular and molecular mediators of neuroinflammation which occur in response to or as part of the ongoing disease progression in PD.

2. Mediators of Neuroinflammation

2.1. Role of Microglia as a Mediator of Neuroinflammation in PD. Glia are composed of three distinct cells types named as microglia, astrocytes, and oligodendrocytes [24]. Several populations of macrophages are present in different compartments of healthy brain tissue, each with a distinct phenotype and morphology. The most abundant of these macrophages are the microglia, the resident macrophages of the brain parenchyma [25]. Microglia maintains homeostasis and performs immune surveillance by continuously examining their environment by extending cellular protrusions [26]. With a plentitude of ion channels, cytokines, Toll-like receptors, and chemokine receptors [21], microglia promptly reacts in response to subtle alterations in their microenvironment such as alterations in ion homeostasis and brain insults, ranging from aggregated proteins to pathogens [27]. Microglial cells are generally quiescent in the normal brain, with their cell bodies barely visible and few detectable fine ramified processes. However, resting microglial cells quickly proliferate, become hypertrophic, and persistently increase expression of a large number of marker molecules such as CD11b, CD68, and MHC-I and II molecules [28] and are further transformed to macrophage-like cells in patients with PD [11, 29, 30]. Microglia may be transformed into M1 or M2 macrophages depending upon the type of stimulus [31, 32]. It is now apparent that microglia occur in many different phenotypes that cannot be readily divided into a small number of discrete subsets following tissue injury [33]. The SN is relatively rich in microglia compared with other brain regions [34, 35]. In addition, a reduced level of intracellular glutathione in the SN dopaminergic neurons makes them much more susceptible to a variety of insults, including activated microglial-mediated injury and oxidative stress [35]. This observation indicates that localization of microglia in the SN predisposes dopaminergic neurons to immunological insult in patients with PD [36].

The selective acute degeneration of dopaminergic cells in the SN can be induced by toxins such as 6-hydroxydopamine (6-OHDA), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), and rotenone. In all of these rodent toxin models, dopaminergic cells of the SN degenerate over a period of a few days. A recent study comparing the commonly used medial forebrain or intrastriatal injection of 6-OHDA showed rapid degeneration induced by the toxin, which was accompanied by activation of microglia as assessed by the upregulation of the complement type 3 receptor [37]. Similarly, MPTP-induced neurodegeneration is associated with activated microglia [38, 39]. It has become increasingly apparent that there are various triggers through which microglia are activated to elicit their neurotoxic response. Interestingly, while these diverse toxins exhibit several mechanisms of microglial

activation, toxin-induced activation of NADPH oxidase is the most common pathway through which microglia exerts neurotoxicity [36]. Apart from this toxin-induced microglial activation, various other triggers involved in microglial activation include immunological insults such as IFN-γ, lipopolysaccharide (LPS), chemokines (CCL5, CCL2, and CXCL10), neurotransmitters, gangliosides, the CD40 ligand, proteases such as thrombin [40], tissue plasminogen activator [41], matrix metalloproteinase-3 (MMP-3) [21], endogenous disease proteins, and neuronal injury itself [42]. Among these activators, LPS-induced neuroinflammation is one of the most accepted and widely used endotoxin models that induces a strong neuroinflammatory response in BV-2 microglial cells [43, 44] or when injected directly into the vicinity of the SN [45]. Recent findings demonstrate that neurons are not simply passive targets of microglia but rather control microglial activation [3, 46, 47]. A variety of signals that neurons use to modulate microglia can be categorized into excitatory and inhibitory signals. Inhibitory signals from neurons constitutively maintain microglia in their quiescent state and antagonize proinflammatory activity, whereas excitatory signals are inducible and incite activation of microglia under pathological conditions towards a beneficial or detrimental phenotype. Thus, various neuronal signaling molecules actively modulate microglial functions and contribute to the inflammatory milieu of PD [46]. The two subclasses of neuronal inhibitory signals are called released and membrane-bound signals. Released inhibitory signals can be CX3CL1, CD22, TGF-β, brain derived neurotrophic factor (BDNF), neurotrophin-3, nerve growth factor, or neurotransmitters. The release site for neuronal inhibitory signals is not yet known but is thought to be related to synaptic activity [48, 49]. Membrane-bound neuronal inhibitory signals consist of several molecules from the immunoglobulin superfamily including CD200 [50], CD22, and CD47, which are expressed or secreted by neurons that bind to receptors on microglia [48, 51, 52]. These immunoreceptors, so-called tyrosine-based inhibitory motif receptors, contain a cytoplasmic motif that inhibits activation of microglia. This mechanism of maintaining microglia in their silent state also depends upon the density of the ligands expressed by neurons, which are reduced during PD and will thus shift the level of inhibitory tone to activation in microglia [25]. In parallel with the inhibitory signals, neuronal excitatory signals are classified into released and membrane-bound signals. The released excitatory signals control various aspects of microglia function and can be listed as chemokines (CX3CL1, CCL21, and CXCL10) [53–55], glutamate [56], purines (ATP and UTP) [57, 58], or MMP-3 [59, 60] which modulate various aspects of microglia function. Taken together, microglia are maintained in a quiescent state under normal physiological conditions by the orchestrated action of neurons and astrocytes; however, microglia are rapidly activated when integrity of a neuron is disrupted in PD, probably as a result of both direct activation signals from neurons or loss of inhibitory signals by neurons [47].

While it is clear that microglia becomes activated upon neuronal damage, proteases known to modify the extracellular matrix (ECM) may also be a critical mechanism through which damaged neurons activate microglia to produce extracellular superoxide. Earlier reports from Chang et al. emphasized the critical role of ECM proteins in the interactions between microglia and neurons [61]. Later, it was found that MMP-3, a proteinase that degrades ECM components, is released following damage to dopaminergic neurons exposed to 1-methyl-4-phenylpyridinium (MPP^+), exerting neurotoxicity to the dopaminergic neurons [59]. It was also observed that exposure of mesencephalic neuron/glia cultures to MPP^+ results in a dose-dependent increase in MMP-3 protein expression, both in conditioned media and in cell lysates, indicating that death of dopaminergic neurons upregulates MMP-3 expression. These data suggest that MMP-3 is a crucial mediator released upon damage to dopaminergic neurons and that it activates microglia to further propagate neuronal cell death in PD [42]. Besides MMP-3, damaged or dying dopaminergic neurons release neuromelanin to activate microglia in the SN of patients with PD [62, 63]. Neuromelanin has the potential to be neurotoxic, because excess neuromelanin inhibits the function of dopaminergic neurons, proteasomes and induces the production of toxic factors such as TNF-α, IL-6, and nitric oxide (NO) [64, 65]. Among the proinflammatory mediators released during microglial activation, some act synergistically to produce inflammation-related neuronal damage. Hence, the identification of several potential mediators of microglia activation has allowed a general classification of how microglia respond to stimuli. Microglia can also be activated by products of the classical complement cascade and by chromogranin A [66, 67], which has been reported to occur in the PD SN [68]. Among the array of mediators released, superoxide is necessary for both the induction and amplification of neurotoxicity in PD [69, 70]. NADPH oxidase (PHOX) is the major superoxide-producing enzyme in microglia [71]. Activation of PHOX results in translocation of its cytosolic subunits to the cellular membrane to form a functional enzyme that not only generates superoxide but also controls the levels of other proinflammatory neurotoxic mediators produced by microglia in PD [72]. It has been revealed that PHOX is closely paired with Mac-1 and plays an important role in microglia-mediated neuroinflammation and neurotoxicity [73]. Therefore, coupling between PHOX and Mac-1 might be a central mechanism responsible for the oxidative damage induced by reactive microgliosis that results in progressive neuroinflammation in PD [36].

To date, one of the best elucidated cytotoxic mechanisms induced by proinflammatory cytokines in PD is activation of iNOS. iNOS mediates the synthesis of high levels of NO, which is toxic to dopaminergic neurons [74]. The density of glial cells expressing iNOS increases significantly in the SN of patients with PD compared with that in control subjects [75]. The induction of NOS produces high levels of NO and superoxide radicals for a prolonged period of time. These two ROS can either directly or indirectly promote neuronal death in PD [76]. Prostaglandins and their synthesizing enzymes, such as COX-2, represent a second group of potential culprits in PD. Expression of COX-2 along with the levels of its product, prostaglandin E2 (PGE_2), increases significantly in glial cells of the SNpc, which are responsible for many of the cytotoxic

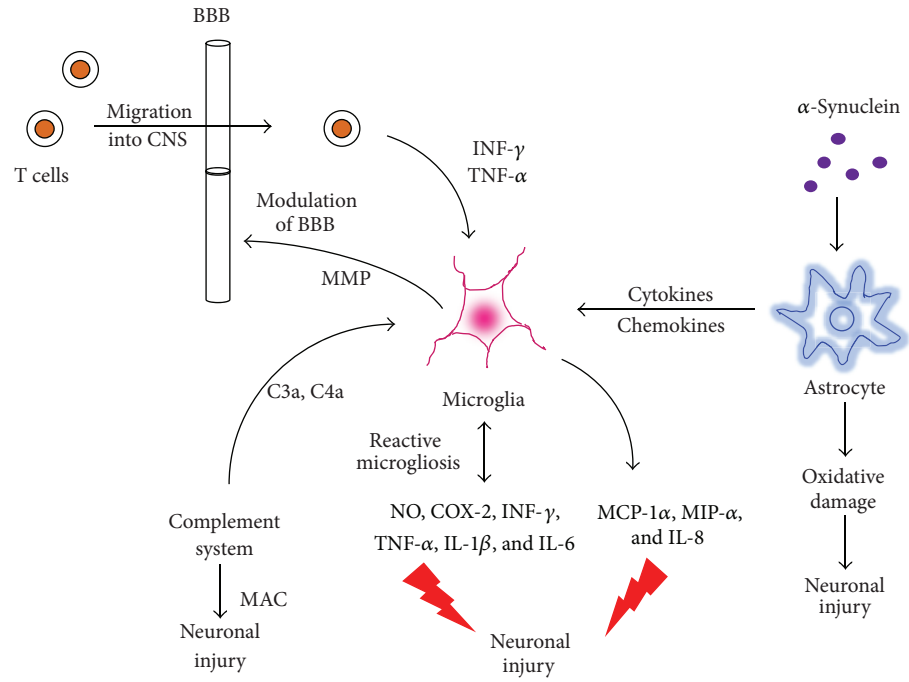

Figure 1: Microglial and astroglial cells become activated during the pathogenesis of Parkinson's disease under the influence of various proinflammatory triggers, including α-synuclein, the complement system, and cytokines released from infiltrated T cells. Activated microglial and astroglial cells further release various neuroinflammatory mediators, including NO, COX-2, IFN-γ, TNF-α, IL-1β & IL-6, chemokines including MCP-1α, MIP-α and CXCL-8, and MAC which have deleterious effect on neuronal survival. Abbreviations: NO: nitric oxide, COX-2: cyclooxygenase, INF-γ: interferon-γ, TNF-α: tumor necrosis factor-α, IL-1β: interleukin-1β, IL-6: interleukin-6, MCP-1α: monocyte chemotactic protein-1, MIP-α: microphage inflammatory protein, IL-8: interleukin-8, MAC: membrane attack complex, α-syn: α-synuclein, MMP: matrix metalloprotein, BBB: blood brain barrier, C3a: complement component 3a, and C4a: complement component 4a.

effects to dopaminergic neurons in PD [77]. Many reports have demonstrated increased expression of COX-2 in PD [77, 78]. In fact, several studies have observed upregulation of COX-2 in animal models of PD [79–81]. Increased COX-2 expression has also been shown in the SN of postmortem PD specimens compared to that in normal controls [75, 82]. Inhibiting COX-2 [80, 81, 83, 84] and transgenic mice lacking COX-2 expression [85] in models of PD has been demonstrated to increase survival of dopaminergic neurons. Release of α-synuclein (α-syn) from neuronal damage could also incite the production of proinflammatory mediators such as PGE_2 from microglia [86], thus, contributing to the progression of nigral neurodegeneration. It has recently been observed that modifying ubiquitin carboxy-terminal hydrolase L1 by cyclopentenone prostaglandins causes protein unfolding and aggregation. Hence, the deleterious effect of COX-2 in PD could be due to the production of cyclopentenone prostaglandins [87]. Although the exact causal link between neuronal injury and microglial activation in PD remains controversial, one of the earliest reported harmful effects demonstrated to cause demise of dopaminergic neurons was microglial-mediated release of proinflammatory cytokines, including IFN-γ [20] IL-1β, TNF-α, IL-2, and IL-6 [88] with elevated levels of TNF-α receptor R1 (p55), bcl-2, soluble Fas, caspase-1 and caspase-3 in postmortem striatum, SN, and CSF of patients with PD [89, 90]. These cytokines, in turn, propagate and intensify neuroinflammation and cause irreversible destruction of SN dopaminergic neurons [91] by a number of mechanisms, including upregulating isoforms of phospholipases A2, generating platelet-activating factor, stimulating NOS, and activating calpain [9, 92]. We have briefly summarized some of the major mediators of neuroinflammation during the pathogenesis of PD in Figure 1.

2.2. Role of T Cells as a Mediator of Neuroinflammation in PD. The brain has long been considered an immune privileged system, as it is protected by the BBB. However, recent findings demonstrate that both the innate and adaptive immune systems play a very critical role disrupting BBB permeability and mediating the pathogenesis of PD via their ability to supply the required signals for antigen presentation and to act as final effectors by T cells [25, 93, 94]. For example, infiltration of T cells has been found in the brains of patients with PD [95] as well as significant infiltration of adoptively transferred immune splenocytes into the brains of MPTP-intoxicated mice and localization within the inflamed SN [93]. Similarly, a recent study of MPTP model demonstrated the necessity of T cells to mediate degeneration of dopaminergic neurons and that dopaminergic neuronal loss is exacerbated by T cells [96]. Increased mutual coexpression of CD4 and CD8 by $CD45R0^+$ T cells with increased expression of CD25, TNF-α receptors, and diminished expression of IFN-γ receptors

suggests that subsets of T cell are indeed activated in patients with PD [97]. The influence of infiltrating T cells on dopaminergic neurons has been demonstrated in mice lacking T lymphocytes, wherein death of dopaminergic neurons was significantly attenuated in both types of mice as compared to that in wild-type animals [22]. It was also observed that a subset of $CD4^+$ T cells, rather than $CD8^+$ T cells, mediate the cytotoxic effects on dopaminergic neurons, as survival of dopaminergic neurons after MPTP administration increases in CD4-deficient mice but not in CD8-deficient mice [22]. Further analysis showed that $CD4^+$ T cells exert their cytotoxicity through the Fas-FasL pathway rather than through IFN-γ secretion [98]. Early evidence from the SN by autopsy of patients with PD showed increased numbers of $CD8^+$ T cells in close proximity with activated microglia and degenerating neurons [99]. More recently, both $CD4^+$ and $CD8^+$ T cells have been discovered within the SN of patients with PD [22]. Significant level of unrepaired single-strand DNA breaks and a number of micronuclei are more also observed in lymphocytes and activated T cells from patients with PD due to inflammation and exposure to ROS than those in age-matched controls [100, 101]. $CD8^+$ T cells were the first type of peripheral T lymphocytes to be located in the postmortem brain from a PD patient [13]. Later, infiltration of $CD4^+$ and $CD8^+$ T cells was found in the SN and striatum of MPTP-intoxicated mice [93, 102]. A more recent report provided substantial evidence of significant infiltration of $CD4^+$ and $CD8^+$ T cells in the SN of patients with PD and in MPTP-intoxicated mice [22]. Increased frequencies of activated $CD4^+$ T cells expressing Fas [103], increased IFN-γ-producing Th1 cells, decreased IL-4-producing Th2 cells, and a decrease in $CD4^+$, $CD25^+$ T cells have been found in the peripheral blood of patients with PD [104]. These data suggest complex roles for $CD4^+$ subsets of T cells in mediating the development of PD.

Compelling evidence suggests the possible involvement of the BBB, including changes in lymphocytic subpopulations in the blood and CSF of patients with PD [91, 103]. Moreover, an increased proportion of $\gamma\delta$-positive lymphocytes, thought to play a role in infections and autoimmunity, have also been reported in the CSF and blood of patients with PD [105]. These data suggest that infiltration of immune cells across the BBB into the brain participates in the pathophysiology of PD. This infiltration of peripheral lymphocytes into the brain through the BBB occurs mainly because activated microglia and monocytes in the brains of patients with PD release proinflammatory cytokines and chemokines that act on the vascular endothelium to induce upregulation of cell adhesion molecules, including vascular cell adhesion molecule-1 and intercellular adhesion molecule-1 (ICAM-1) [106] that disrupt the BBB and attract lymphocytes [97] expressing counter-receptors such as leukocyte function antigen-1 (LFA-1) to the neuronal injury site [106]. Activated T cells further release proinflammatory cytokines such as IFN-γ and TNF-α, which positively induce the expression of costimulatory molecules and MHC-II on microglial cells [107]. When these activated microglial molecules bind to their respective receptors on T cells, they are transformed into effector T cells. Among the activated T cells, $CD8^+$ T lymphocytes mediate direct cytotoxic lysis of target cells, recruit and activate accessory cells via proinflammatory mediators, whereas activated $CD4^+$ T lymphocytes induce B cells to produce high-affinity antibodies [91]. Nitrated-α-syn (N-α-syn), a misfolded protein present in intraneuronal inclusions or Lewy bodies in patients with PD, can be released to the extraneuronal space and cross the BBB to enter the cervical lymph nodes, where it can activate antigen-presenting cells [93]. This phenomenon has also been observed in MPTP-intoxicated mice, wherein α-syn drains to cervical lymph nodes [93]. As a novel epitope, N-α-syn can be processed and presented to naive T cells, thus, stimulating them to expand into different subsets of effector T cells. Peripherally activated effector T cells, such as Th1 or Th17 cells, can also cross the BBB and reach inflammatory sites within the brain where they activate microglia and release proinflammatory cytokine IL-17 and secrete cytolytic enzymes such as granzyme B [108]. Although a dysfunctional BBB in patients with PD may show some leakiness [109], it may not be sufficient to allow unrestricted lymphocyte infiltration because CD4/CD8 ratios are 1 : 4.8 [22] compared with the typical 2 : 1 ratio expected for peripheral T cells performing surveillance functions. Thus, the mechanisms by which these T cells gain access to the SN, their activation state, and their functions are questions that remain to be answered [97].

In summary, infiltrating subsets of T cells may induce excessive microglial-mediated inflammation and oxidative stress that exacerbate neuroinflammation in PD. Further study is needed to identify the exact roles of specific subsets of $CD4^+$ T cells on the pathogenic progression of PD [98]. Studies suggest that the adaptive immune system similar to the innate immune system not only responds to but also actively participates in the pathogenesis of PD. However, more work needs to be done to determine if and how they could serve as a potential target for PD therapy [36].

2.3. Role of Astrocytes as a Mediator of Neuroinflammation in PD. The majority of findings from research on PD point toward microglia as the major mediator of neuroinflammation, but the astrocytic reaction is another well-known neuropathological characteristic in the SN of patients with PD [110]. Astrocytes function as supportive cells for neurons and maintain homeostasis and other neuronal functions [111]. Compared to microglia, their role as innate immune cells is somehow less appreciated. Nonetheless, astrocytes form the glia limitans around blood vessels, preventing entry of immune cells via the BBB into the CNS parenchyma [112]. Emerging evidence has focused upon the importance of astrocytes in the regulation of neuroinflammation in PD [113, 114]. The SNpc of many postmortem PD cases had been observed to have an increased number of astrocytes and GFAP immunoreactivity [115] with a decrease in glial-derived neurotrophic factor, BDNF, and ciliary neurotrophic factor [116]. The amount of GFAP-positive astrocytes is inversely proportional to the demise of dopaminergic neurons [12], indicating that dopaminergic neurons are more susceptible to the degenerative process wherein there are fewer astrocytes. Activation of astrocytes is characterized by the formation of hypertrophic and glial scars, which hinder axonal

regeneration [42], enlarged cell bodies, and projections into the injured area [117] that seem to be mediated by proteoglycans [118]. During inflammatory conditions, astrocyte-derived granulocyte macrophage-colony stimulating factor, IL-6, CCL2, and CCL5 regulates migration, activation, and proliferation of microglia [119]. Astrocytes may detect neuron-derived α-sys as a degenerative marker released by neurons and get activated to protect neurons. However, such reactive astrocytes are exposed to increasing toxicity from α-sys oligomers and/or protofibrils, until they no longer serve a protective function [120].

Astrocytes play a major role mediating MPTP toxicity, as the active metabolite of MPTP, MPP^+ is extruded into the extracellular space from astrocytes and further enters into dopaminergic neurons and induces neurotoxicity by inhibiting complex I in the mitochondrial electron transport chain [113]. Astrocytic activation parallels the development of dopaminergic cell death in the SNpc and striatum, whereas GFAP expression remains high even after most dopaminergic neurons have died due to administration of MPTP. These findings suggest that the astrocytic reaction occurs after neuronal cell death in PD [121]. In a recent study, it was demonstrated that α-syn released from neuronal cells can also be transferred to and accumulate in astrocytes and induce expression of genes associated with immune functions [122]. Proinflammatory cytokines that are differentially expressed in astrocytes in response to extracellular α-syn include IL-1α, IL-1β, IL-6, IL-18, and colony-stimulating factors-1, 2, and 3, suggesting a strong inflammatory response from astrocytes upon exposure to neuron-derived α-syn [122]. Exposure to neuron-derived α-syn also causes dramatic changes in chemokine expression in astrocytes, including CC-type (CCL-3, 4, 5, 12, 20), CXC-type (CXCL-1, 2, 5, 10, 11, 12, 16), and CX3C-type (CX3CL1) chemokines. These chemokines are involved in a variety of functions, such as recruitment of monocytes and macrophages, migration of microglia and neural progenitors, regulation of microglial activity, proliferation and survival of astrocytes, and synaptic plasticity and transmission [123]. These released chemokines, including CXCL12 and CCL5, induce glutamate release and restart the synthesis of cytokines and chemokines in astrocytes, suggesting their role in glia-glia and glia-neuron communication [122].

Other than cytokines, inflammatory oxidants have emerged as key contributors to PD and MPTP-related neurodegeneration. In this context, myeloperoxidase (MPO), a key oxidant-producing enzyme, which is mostly expressed by reactive astrocytes during inflammation, is upregulated in the ventral midbrain of human patients with PD and in MPTP mice [110]. MPO oxidizes nonreactive nitrite whose concentration is increased in parkinsonism [124] to reactive nitrite (NO_2^-) and, thus, nitrosylates many proteins [125]. Reactive nitrites also contribute towards the production of the nonradical oxidant, hypochlorous acid (HOCl), which can damage macromolecules indirectly by fuelling hydroxyl freeradicals or directly by converting amines into chloramines, phenols, and unsaturated bond chlorination [126]. Furthermore, MPO also directly produces HOCl from hydrogen peroxide and chloride anion. Thus, HOCl might directly inflict oxidative damage on dopaminergic neurons [110]. Apart from the direct release of proinflammatory cytokines, including TNF-α and IL-6, astrocytes can also be activated by cytokines such as IL-1β and TNF-α released from microglia, thus, producing ROS and RNS [127]. In support of this observation, a recent study has reported that microglial inflammatory responses are enhanced by astrocytes through a nuclear factor-κB-dependent mechanism leading to increased dopaminergic toxicity [127]. Astrocytes also produce mediators which play a vital role mediating the inflammatory reaction that occurs in the SN of patients with PD. For example, ICAM-1-positive astrocytes are seen in the SN in patients with PD and attract reactive microglia to the area because such microglia carries the LFA-1 counterreceptor [95]. In addition, α-syn activates microglia and astrocytes to produce IL-6 and ICAM-1 [128]. This combination further attracts reactive microglia to the site of neuronal injury. The action of α-syn on astrocytes is thought to be through receptors, but the identity of these receptors is currently unknown [3].

Activated $CD4^+$ T cells express and release several inflammatory factors such as the Fas ligand, a cell-surface molecule in the TNF-α family. Fas expression increases in patients with PD and in mice exposed to MPTP [129, 130]. This Fas ligand binds with the Fas receptor expressed on astrocytes and causes a release of various cytokines such as IL-6 and IL-8 and chemokines such as monocyte chemoattractant protein-1 [110]. The detrimental consequence of activating the Fas-Fas ligand pathway in PD has recently been established by several investigators. It has also been reported that mice deficient in Fas are more resistant to MPTP exposure than wild-type controls [131]. The current literature certainly suggests that astrocytes have the ability to modulate the function and survival of dopaminergic neurons in PD.

2.4. Role of the Complement System as a Mediator of Neuroinflammation in PD. The complement system is believed to amplify the effectiveness of both the specific and nonspecific immunological defense system. The complement system destroys invading pathogens, encourages inflammation, and supports phagocytosis of waste materials [132]. The complement system has the full ability to recognize molecular patterns associated with injured tissues and dying cells or molecular patterns on pathogens [133]. Various complement proteins, mostly present in tissue fluids and blood, are in the form of soluble monomers. The complement system can be activated by molecules other than antibodies. One such molecule, which is elevated in the SN of patients with PD, is C-reactive protein [134]. Similar evidence about the involvement with the complement system in PD has also been reported wherein membrane attack complex (MAC) together with all complement protein components has been identified on oligodendroglia of the SN and intracellularly in Lewy bodies of patients with sporadic PD [135, 136] and familial PD [137]. Additionally, elevated levels of MAC [135], C-reactive protein, and complement 3 have been observed in the SN and CSF of patients with PD [138, 139]. Increased mRNA levels of complement components have also been found in affected brain regions of PD models [138]. Activation of the

complement system leads to a cascade of events ultimately leading to the destruction of cell surface with three different recognized pathways, which share a common juncture at the level of the C3 protein [140, 141].

Products of the activated complement cascade include opsonizing components (C3b, iC3b, and C4b) [142], which stain material for phagocytosis, MAC, and anaphylatoxins (C3a, C4a, and C5a) [142]. The opsonins perform a clearance function, whereas anaphylatoxins are involved in generation of the neuroinflammatory response [143]. In contrast, MAC induces cell death by entering cell membranes and causing organelles to leak. Although MAC destroys foreign cells and viruses, nearby host cells are at a significant risk of lysis if they are not protected by MAC [135, 140]. The complement system also contributes to the secretion of inflammatory cytokines from activated microglial cells [144]. Very recent evidence has demonstrated involvement of the complement system in the pathogenesis of PD, wherein the only cells in the SN and other brain areas that express C1q are microglia [145]. One of the important features of PD is that degeneration of dopaminergic neurons is accompanied by the deposition of extracellular neuromelanin. Degenerating neurons along with neuromelanin granules are opsonized by C1q and phagocytosed by C1q-positive microglia and macrophages in the perivascular spaces and parenchyma. Furthermore, the luminal surfaces of blood vessels in the SN of patients with PD have attached neuromelanin-laden C1q-positive cells. Thus, microglia are capable of clearing cellular debris from degenerating neurons of the SN and phagocytosing cells through the C1q-mediated pathway in PD [145]. Pentraxin is one of the mediators which activates the complement system by binding to the collagen tail of C1q. Pentraxin is an acute-phase protein that is involved in innate immunity and inflammatory response. Glial cells may be the major cellular source of this protein in the CNS. Under the inflammatory milieu of PD, pentraxin proteins secreted by reactive glial cells are detected in the plasma and CSF of patients with PD [146, 147]. Hence, pentraxin could serve as an inflammatory biomarker for PD. Overall, it seems clear that there is a role for the complement system in inflammation-mediated neurodegeneration in PD [138, 140]; hence, research aiming at developing effective inhibitors targeting these sites appears to be worthwhile.

3. Conclusion

PD is one of the most common neurodegenerative diseases with a well-established group of symptoms. Although a number of different mechanisms have been considered responsible for the development of PD, none are absolute. Growing evidence from patients and experimental models of PD has indicated that neuroinflammation is one of the driving forces in the pathogenesis of PD. The CNS had been thought to be an immunologically protected organ, but this notion has now undergone considerable reassessment. It has become apparent from a number of reports that various neuronal injury signals from different neuronal cell types in response to environmental insults, involving many mediators, incite and disseminate the ongoing neuroinflammation in PD. We have summarized the evidence wherein neuroinflammatory mediators play a key role in the pathogenesis of PD. Neuroinflammatory mediators have a profound action on CNS cells that differently affect the progress of inflammation and neuronal death. Therefore, regulating the production of neuroinflammatory mediators or their action on respective receptors would be an effective approach to mitigate the inflammatory processes in PD. Thus, further studies are required to form a more comprehensive idea about the role of these neuroinflammatory mediators in PD. Furthermore, it is of significant interest for ongoing research to identify and target various neuroinflammatory mediators released in response to various toxins to help explain how neuronal damage can signal inflammation and propagate neuronal cell death. This knowledge might serve to develop pharmacological strategies for treating the neuroinflammation in PD.

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Research Article

Persistent Inflammation in the CNS during Chronic EAE Despite Local Absence of IL-17 Production

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Experimental autoimmune encephalomyelitis (EAE) is an artificially induced demyelination of the central nervous system (CNS) that resembles multiple sclerosis in its clinical, histopathological, and immunological features. Activated Th1 and Th17 cells are thought to be the main immunological players during EAE development. This study was designed to evaluate peripheral and local contribution of IL-17 to acute and chronic EAE stages. C57BL/6 mice were immunized with MOG plus complete Freund's adjuvant followed by pertussis toxin. Mice presented an initial acute phase characterized by accentuated weight loss and high clinical score, followed by a partial recovery when the animals reached normal body weight and smaller clinical scores. Spleen cells stimulated with MOG produced significantly higher levels of IFN-γ during the acute period whereas similar IL-17 levels were produced during both disease stages. CNS-infiltrating cells stimulated with MOG produced similar amounts of IFN-γ but, IL-17 was produced only at the acute phase of EAE. The percentage of Foxp3+ Treg cells, at the spleen and CNS, was elevated during both phases. The degree of inflammation was similar at both disease stages. Partial clinical recovery observed during chronic EAE was associated with no IL-17 production and presence of Foxp3+ Treg cells in the CNS.

1. Introduction

Multiple sclerosis (MS) is a progressive inflammatory disorder of the central nervous system (CNS) that chronically affects both brain and spinal cord. The pathological hallmark of this pathology is an inflammatory plaque that can be detected by histopathological analysis and, more recently, by using magnetic resonance imaging [1–4]. Findings derived from both patients and animals studies indicated the presence of inflammatory cells and their products raising the general accepted hypothesis that this pathology is mediated by myelin self-reactive T cells [5, 6]. These self-aggressive T cells reach the CNS by crossing the blood-brain barrier (BBB) [7, 8]. At the CNS they destroy the myelin sheet leading to signal conduction slowing or even signal block at the site of inflammation [9].

Experimental autoimmune encephalomyelitis (EAE) is an artificially induced demyelination disease of the CNS that resembles MS in its clinical, histopathological, and immunological features [10]. It is induced in susceptible laboratory animals by immunization with proteins from the CNS, such as myelin proteolipid protein, myelin oligodendrocyte glycoprotein (MOG), or myelin basic protein associated with complete Freund's adjuvant (CFA) [11–13]. This experimental disease is also mediated by myelin-specific T cells, which are initially activated at the peripheral lymphoid organs and then reach the CNS by crossing the permeabilized BBB [7, 14]. Depending upon the mice strain and the immunization strategy, EAE will present different courses, portraying an acute, chronic progressive, or relapsing-remitting kind of disease [9, 15]. The EAE model has indubitably provided a lot

of information about the inflammatory processes throughout the progression of the disease. MOG-induced EAE in C57BL/6 mice is amongst the most frequently used mouse models for MS studies [10, 16]. Much of our actual knowledge came from investigations done with EAE in mice. Briefly, activated Th1 and Th17 cells are thought to be the main immunological players during EAE and MS development. Many of their effects would be mediated by IFN-γ and IL-17, respectively. They would be initially primed by contact with CNS-specific antigens presented by antigen presenting cells (APCs) in peripheral lymphoid organs. These T cells would then cross the BBB and, by recognizing their cognate antigen presented by CNS resident or immigrating APCs, would initiate a local inflammatory process that would, ultimately, destroy myelin and axons [10, 15]. Which subset of helper T cells is most critical for the pathogenesis of EAE is still a subject of intense controversy. Mice deficient in either RORγt or T-bet are resistant to EAE induction, supporting the opinion that both Th17 and Th1 cells participate in CNS autoimmune pathologies [17, 18]. Association of techniques that allow purification of brain infiltrating cells and FACS analysis have contributed a lot to elucidate the participation of T cell subsets in EAE development [19, 20]. Collectively the findings obtained with these methodologies demonstrated that both autoreactive Th1 and Th17 cells, their balance at the site of inflammation, and their cytokines and chemokines are responsible for CNS autoimmunity. Studies analyzing the phenotype of T cells infiltrating the CNS during EAE revealed the presence of both Th1 and Th17 cells [21, 22]. However, there was an interesting difference in their proportions when distinct mice lines were compared. For example, C57BL/6 mice showed Th1 predominance whereas SJL mice displayed more Th17 in the inflamed CNS during higher clinical scores [21, 22]. The immunization procedure also significantly affected this differential contribution of Th1 and Th17 cells. Mice immunization with similar but distinct MOG epitopes evoked T cell responses characterized by different Th1/Th17 proportions, depending on the avidity of T cells for their corresponding epitopes [23]. It has been hypothesized that this differential ratio of Th1 and Th17 cells in CNS-infiltrating cells could explain the variety of clinical disease manifestations found in MS patients [24]. In this context, the present study was designed to evaluate the peripheral and local contribution of IL-17 to acute and chronic EAE stages in C57BL/6 mice.

2. Material and Methods

2.1. Animals. Female C57BL/6 mice (8–10 weeks old) were purchased from CEMIB (UNICAMP, São Paulo, SP, Brazil). The animals were fed with sterilized food and water *ad libitum* and were manipulated in accordance with the ethical guidelines adopted by the Brazilian College of Animal Experimentation. All experimental protocols were approved by the local Ethics Committee (Ethics Committee for Animal Experimentation, Medical School, Univ. Estadual Paulista).

2.2. EAE Induction. MOG35–55 peptide (MEVGWYRSPFSRVVHLYRNGK) was synthesized by Proteimax, São Paulo, Brazil. EAE was induced as previously described [20]. Briefly, mice were immunized subcutaneously with 150 μg of MOG35–55 peptide emulsified in CFA containing 5 mg/mL of mycobacteria. Mice also received 2 doses, 0 and 48 h after immunization, of 200 ng of *Bordetella pertussis* toxin (Sigma) intraperitoneally. Clinical assessment of EAE was daily performed according to the following criteria: 0—no disease, 1—limp tail, 2—weak/partially paralyzed hind legs, 3—completely paralyzed hind legs, 4—complete hind and partial front leg paralysis, and 5—complete paralysis/death.

2.3. CNS-Infiltrating Cells Isolation. Mice were anesthetized with ketamine/xylazine and perfused with 10 mL of saline solution. Brain and cervical spinal cords were excised, macerated, and maintained in 4 mL of RPMI (Sigma) supplemented with 2.5% collagenase D (Roche) at 37°C, 5% CO_2 incubator. Forty-five min later suspensions were washed in RPMI and centrifuged at 450 ×g for 15 min at 4°C. Cells were resuspended in percoll (GE Healthcare) 37% and gently laid over percoll 70% in tubes of 15 mL. The tubes were centrifuged at 950 ×g for 20 min with centrifuge breaks turned off. After centrifugation the ring containing mononuclear cells was collected, washed in RPMI, and centrifuged at 450 ×g for 5 min. Cellular suspensions were then resuspended in complete RPMI medium, counted, and analyzed.

2.4. Cell Culture Conditions and Cytokine Quantification. Control and EAE mice were euthanized 19 days (acute phase) or 30 days (chronic phase) after EAE induction. Lymph nodes (popliteal + inguinal), spleen and CNS-isolated cells were collected and adjusted to 2.5×10^6 cells/mL, 5×10^6 cells/mL, and 2.5×10^5 cells/mL, respectively. Cells were cultured in complete RPMI medium (RPMI supplemented with 5% of fetal calf serum, 20 mM glutamine, and 40 IU/mL of gentamicin). Spleen and lymph node cells were stimulated with MOG (20 μg/mL) and Concanavalin A, Sigma Aldrich (10 μg/mL). CNS-isolated cells were restimulated *in vitro* with 50 μg/mL of MOG. Cytokine levels were evaluated 48 h later by enzyme-linked immunosorbent assay (ELISA) in culture supernatants using IFN-γ and IL-10 BD OptEIA Sets (Becton Dickinson) and IL-6, IL-17, and TNF-α Duosets (R&D Systems, Minneapolis, MN, USA). The assays were performed according to the manufacturer's instruction.

2.5. Proportion of CD4+CD25+Foxp3+ T Cells. Spleen cells were collected and the red blood cells were lysed with Hank's buffer containing NH_4Cl. Cells from spleen and cervical spinal cord were obtained as described before and adjusted to 2.5×10^6 cells/100 μL. Spleen and CNS-infiltrating cells were then incubated with 0.5 μg of fluorescein isothyocianate (FITC) anti-mouse CD4 (clone GK1.5) and 0.25 μg of allophycocyanin (APC) anti-mouse CD25 (clone PC61.5) for 20 min at room temperature. A staining for Foxp3 was then performed utilizing the phycoerythrin (PE) anti-mouse/rat Foxp3 Staining Set (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions. After incubation, the cells were fixed in paraformaldehyde 1%. The cells were analyzed by flow cytometry using the FACSCalibur

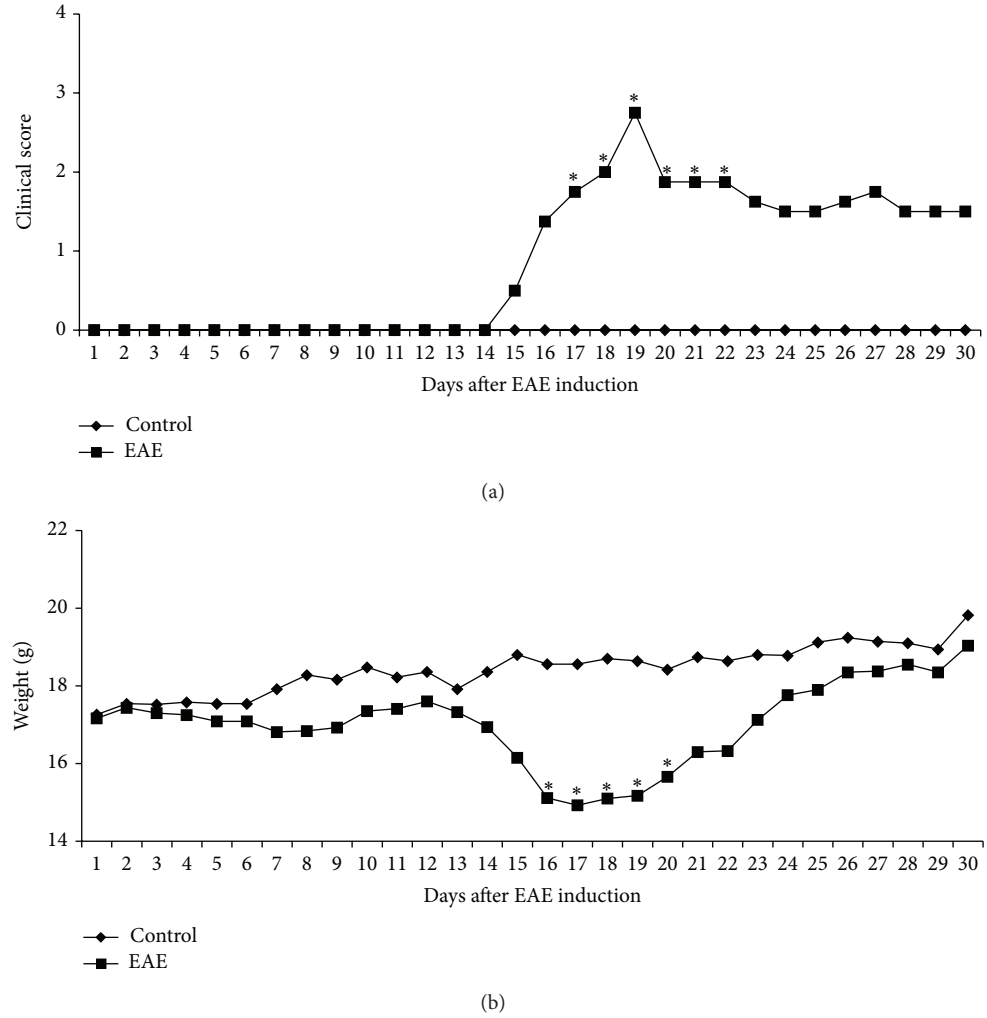

Figure 1: Female C57BL/6 mice were submitted to EAE induction by immunization with MOG emulsified in CFA. Clinical score (a) and weight variation (b) were daily evaluated during 30 days. Data were presented by mean ± SE of 6 mice and representative of two independent experiments. $^{*}P < 0.05$.

(Becton Dickinson, San Jose, CA, USA) and BD CellQuest Pro software (Becton Dickinson, San Jose, CA, USA).

2.6. Evaluation of Inflammatory Infiltrates in the CNS. A histological analysis was performed in the CNS at the 30th day after EAE induction. After euthanasia and blood withdrawal, brain and lumbar spinal cord samples were removed and fixed in 10% formaldehyde. Tissues were dehydrated in graded ethanol and embedded in a 100% paraffin block. Serial sections with 5 μm thickness were cut and stained with hematoxylin and eosin. Five to six photos were obtained by each animal with a Nikon microscope.

2.7. Statistical Analysis. Data were expressed as mean ± SE. Comparisons between groups were made by Student's t test or one way ANOVA with post hoc Holm-Sidak test for parameters with normal distribution and by Mann-Whitney U test or Kruskal-Wallis test for parameters with non-normal distribution. Significance level was $P < 0.05$. Statistical analysis was accomplished with SigmaStat for Windows v 3.5 (Systat Software Inc).

3. Results

3.1. EAE Progression. MOG-immunized C57BL/6 mice developed the first signs of EAE around 15 days after immunization by displaying loss of tail tonus. The maximal clinical symptomatology, that is indicative of the acute phase, occurred at day 19 when the average clinical score reached 2.8 (Figure 1(a)). From this period on the animals slightly improved their mobility but did not completely recover from paralysis. The clinical scores that declined to an average of 1.5 did not significantly change until the 30th day that was chosen as the end point of the experiment. Variation in body weight showed an expected course characterized by a significant weight drop during the acute phase (Figure 1(b)). This loss was followed by a progressive weight recovery. Animals with

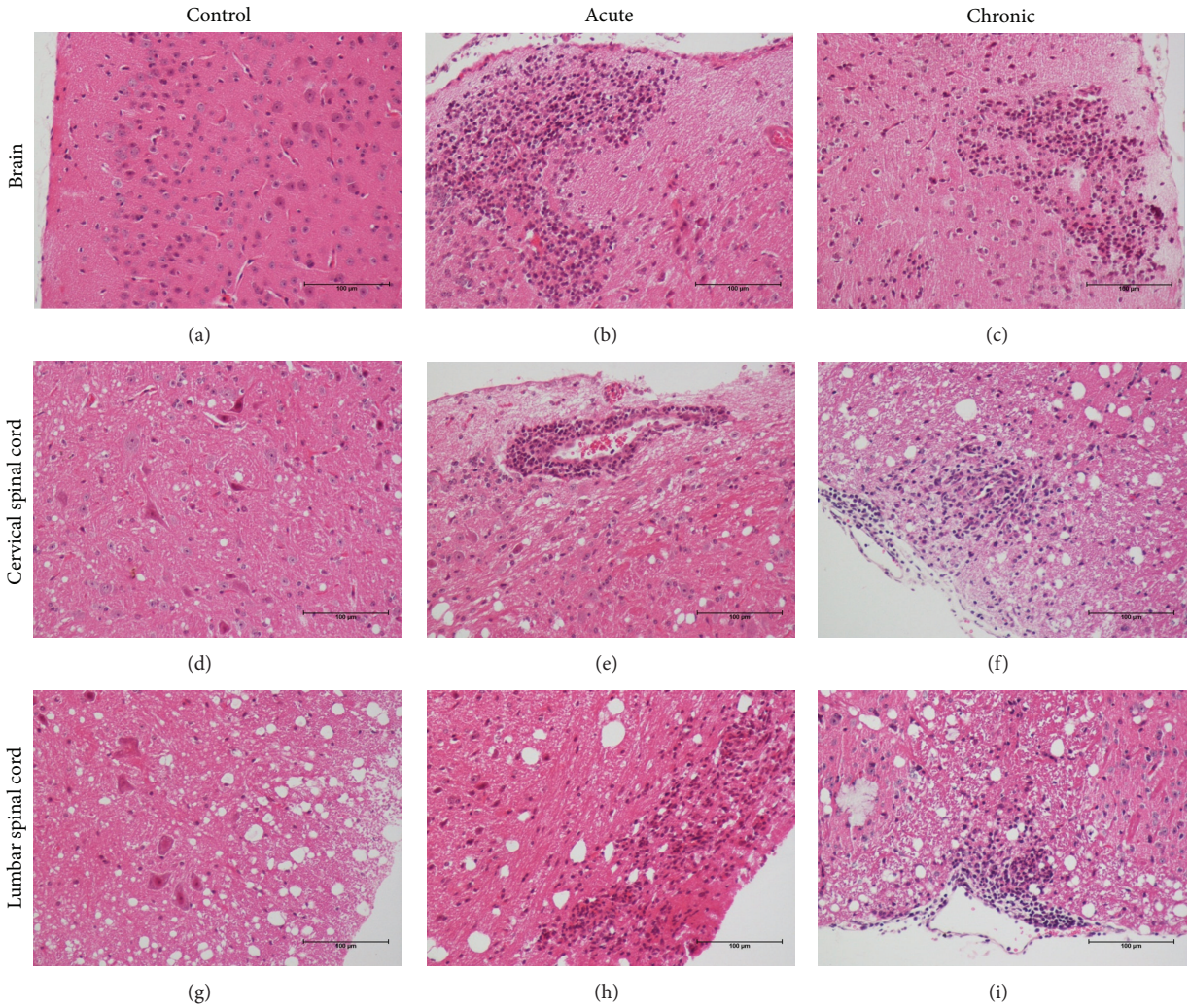

Figure 2: Histopathological analysis of the CNS in C57BL/6 mice with EAE. Female mice were submitted to EAE induction by immunization with MOG emulsified in CFA. Inflammatory infiltrates were evaluated in brain (a, b, and c), cervical (d, e, and f), and lumbar (g, h, and i) spinal cord sections stained with H&E in control animals (a, d, and g) and in animals with EAE during acute (b, e, and h) and chronic disease stages (c, f, and i). Panel is representative of 6 animals/group.

EAE reached weight values similar to the normal control group at the 30th day following immunization.

3.2. Histological Analysis of Brain and Spinal Cord. The histological analysis was performed in samples obtained during the acute and chronic phases. Typical lesions, characterized by an intense perivascular inflammatory infiltrate were observed in the brain (Figures 2(a), 2(b) and 2(c)) and also in both, cervical (Figures 2(d), 2(e) and 2(f)) and lumbar sections (Figures 2(g), 2(h) and 2(i)) of the spinal cord. A visual inspection indicated that the degree of inflammation was equivalent in these two clinical disease phases.

3.3. Production of Cytokines by Peripheral Lymphoid Organs. Cytokine production by peripheral lymphoid organs was compared during acute and chronic phases of the disease. The profile of cytokine production induced by MOG was very similar in spleen and lymph node cell cultures. Elevated levels of IFN-γ (Figures 3(a) and 3(e)), TNF-α (Figures 3(b) and 3(f)), and IL-10 (Figures 3(d) and 3(h)) were present in both phases; however, their values were significantly higher in cultures from acute phase animals. IL-17 (Figures 3(c) and 3(g)) was also elevated during the acute phase but, differently from the other cytokines, its levels remained elevated during the chronic period, presenting no statistical difference in comparison to the acute phase of the EAE.

3.4. Production of Cytokines by CNS Infiltrating Cells. Cytokine production by cells eluted from the CNS stimulated with MOG presented a different behavior, depending upon the cytokine that was being analyzed. TNF-α (Figure 4(b)) and IL-6 (Figure 4(d)) were significantly higher during the acute phase in comparison to the chronic period of the disease. Detectable levels of these cytokines were also present in nonstimulated cultures. IL-10 production showed

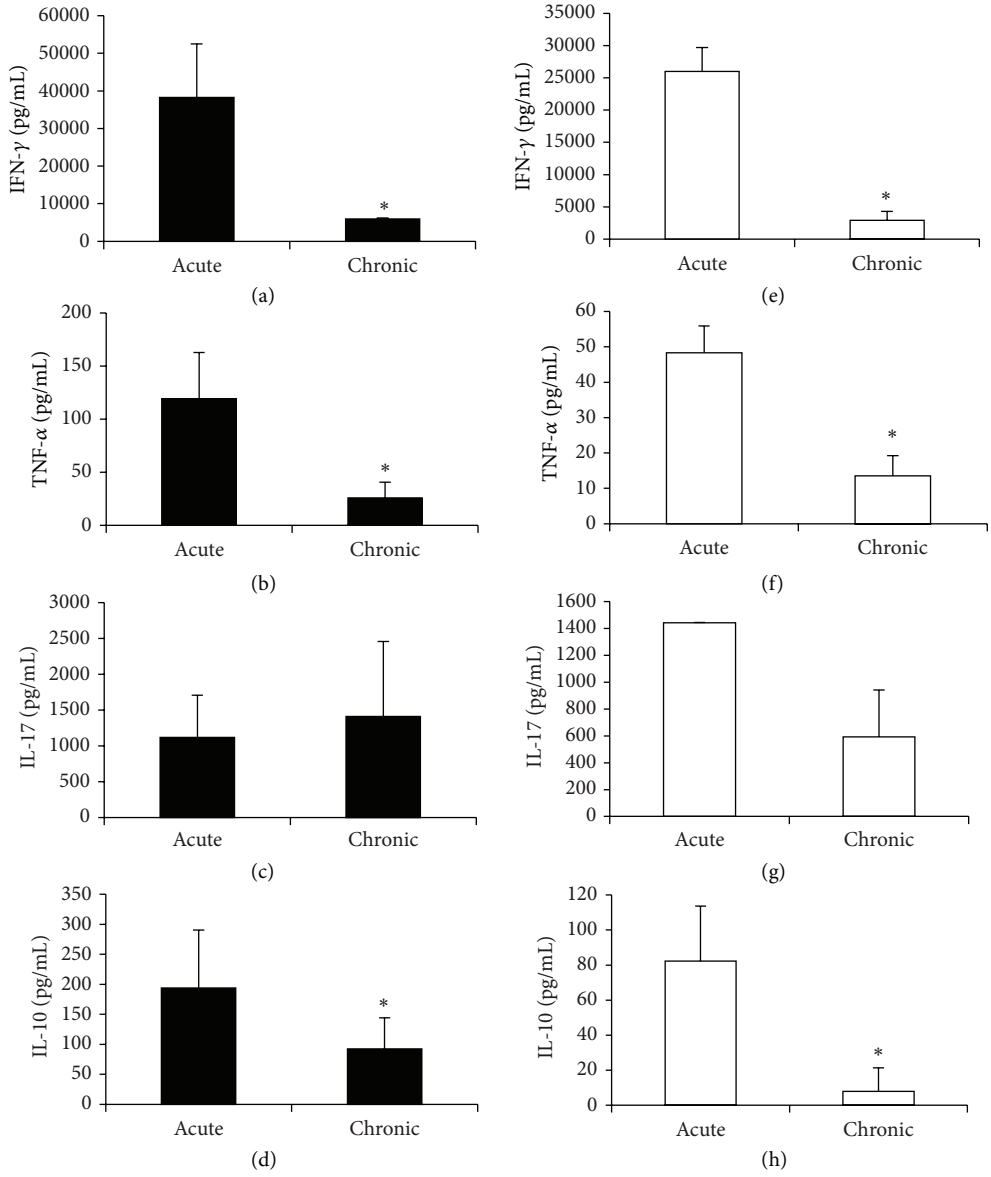

Figure 3: Production of cytokines by peripheral lymphoid organs. C57BL/6 mice were submitted to EAE induction and cytokine production was assayed in acute and chronic EAE stages. IFN-γ (a and e), TNF-α (b and f), IL-17 (c and g), and IL-10 (d and h) levels were measured in spleen (a, b, c, and d) and lymph node cell (e, f, g, and h) cultures stimulated with MOG. Data were presented by mean ± SE of 6 mice and representative of two independent experiments. $^{*}P < 0.05$.

a similar profile with significantly higher production during the acute phase; however, in this case the spontaneous production of this cytokine was very high approaching the levels found in MOG stimulated cultures (Figure 4(e)). IFN-γ levels were, differently from the other cytokines, similarly elevated in both phases with no spontaneous release in culture (Figure 4(a)). IL-17 production presented a completely distinct profile characterized by significantly elevated levels at the acute disease and no production at the chronic phase (Figure 4(c)).

3.5. Quantification of Foxp3+ T Cells. The frequency of CD4+CD25+Foxp3+ T cells was investigated at the spleen and also at the mononuclear cells eluted from the CNS tissue. As can be observed in Figure 5(a), there was a small but significant increase in the Foxp3+ T cell subset, in the spleen, during both phases of EAE development in comparison to normal animals. The analysis made in the CNS cells also revealed the presence of this regulatory T cell subset in both phases (Figure 5(b)). However, differently from the findings in the periphery, there was a drop in Foxp3+ T cells during the chronic EAE phase.

4. Discussion

The main goal of this study was to compare the contribution of IL-17 and IFN-γ to inflammation observed during acute and chronic EAE. The relevance of this investigation resides

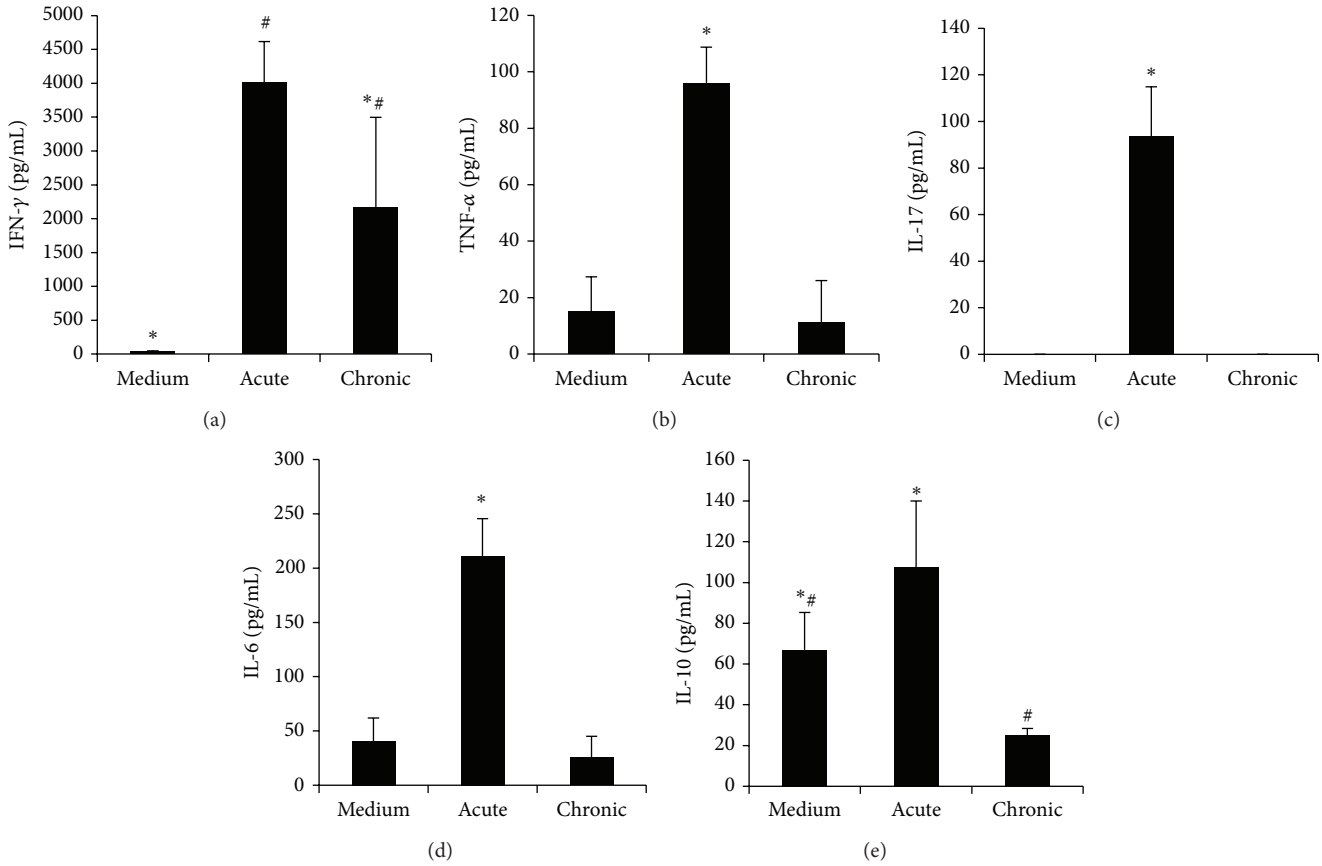

Figure 4: Production of cytokines by CNS infiltrating cells. Female C57BL/6 mice were submitted to EAE induction by immunization with MOG emulsified in CFA and the cytokine production was assayed in acute and chronic stages of EAE. IFN-γ (a), TNF-α (b), IL-17 (c), IL-6 (d), and IL-10 (e) production by cells eluted from the brain stimulated *in vitro* with MOG. Data were presented by mean ± SE of 6 mice and representative of two independent experiments. * and $^{\#}P < 0.05$.

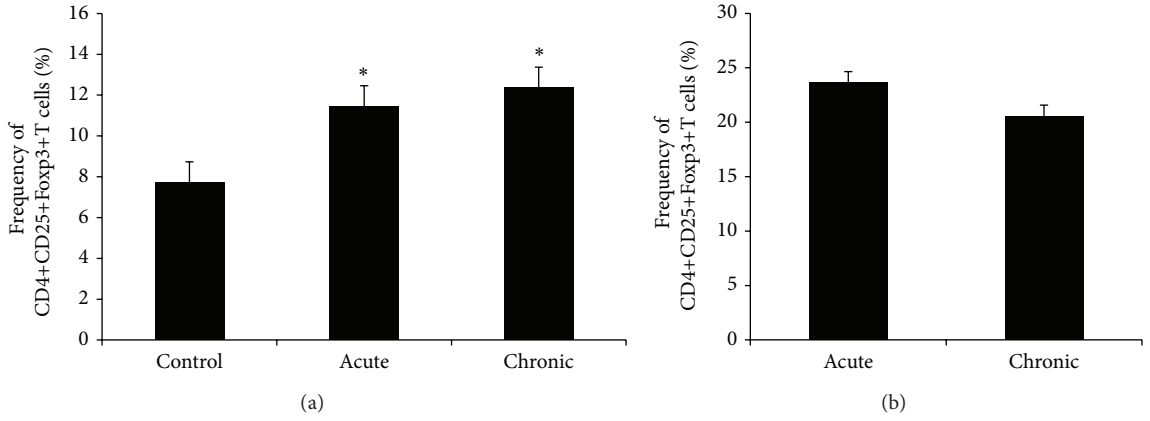

Figure 5: Frequency of CD4+CD25+Foxp3+ T cells in spleen (a) and in CNS (b). The percentage of CD4+CD25+Foxp3+ T cells was determined during acute and chronic stages of EAE by flow cytometric analysis. Data were presented by mean ± SE of 6 mice and representative of two independent experiments. $^{*}P < 0.05$.

in the fact that IL-17 is a relatively recent described cytokine whose role in autoimmune pathologies, including EAE, is not entirely known [10]. In addition, it is highly possible that the relative proportion of IL-17 and IFN-γ at the CNS is associated with the variety of the human disease clinical manifestations [25].

In preliminary assays we established that immunization of female C57BL/6 mice with MOG associated with CFA triggered a classical EAE disease, characterized by ascending paralysis. Two very distinct disease stages were observed: an acute phase and a chronic phase. The acute phase was characterized by the highest clinical scores and the most

significant weight loss. The disease peak (around the 19th day) was followed by a slow drop in clinical score that stabilized around the 30 day after immunization. This second period that was characterized by lower clinical scores and a complete body weight recovery was understood as the chronic EAE stage. A plethora of experimental models is being explored to unravel the immunopathogenetic mechanisms responsible for MS beginning and progression [26]. The model used by us allowed a clear differentiation between acute and chronic phases as has already been described [27]. On the other hand, other authors described a different profile, with no distinction between these two phases [28].

All the following approaches were done with the purpose to compare these two EAE stages. The clinically most severe acute period was concomitant with a higher production of IFN-γ, TNF-α, and IL-10 by peripheral lymphoid organs, compared to the chronic phase. Interestingly and differently from these cytokines, the production of IL-17 remained as elevated in the chronic phase as it was during the acute period. This differential production of IFN-γ and IL-17 during chronic EAE could be explored as a marker to follow MS evolution and, maybe, as an indicator for treatment efficacy. The lower production of some cytokines during the chronic period could result from induction of cells able to regulate the immune response. In accordance with this possibility, more elevated levels of regulatory Foxp3+ T cells were found in the spleen of diseased animals, in both phases, in comparison to normal control mice. Induction, expansion, and maintenance of a putative population of Treg cells have been intensely investigated in EAE [29]. However, this seems to be the first report comparing Foxp3 T cell levels during these two disease phases.

Unexpectedly, the lower clinical scores and decreased cytokine production in the periphery during the chronic period were not associated with a significant decrease in the CNS inflammatory reaction. A visual analysis of the inflammatory infiltrates at both brain and spinal cord (cervical and lumbar) did not reveal a great difference in the degree of cellular infiltration. The most immediate explanation for these findings would be a qualitative difference in these inflammatory infiltrates, rather than the expected downregulation of inflammation during the chronic phase. To confirm this hypothesis we evaluated cytokine production by cells infiltrated in the brain tissue. Production of cytokines by cells isolated from the CNS has greatly contributed to clarify the participation of these molecules in EAE and MS pathologies. This technique allows the most direct and desirable investigation of what happens *in situ*, in the focus of inflammation and demyelination [30]. The local production of some cytokines presented a pattern that resembled the one produced by peripheral cells. TNF-α, IL-6, and IL-10 were found in much higher levels during the acute phase. However, IFN-γ and IL-17 presented very distinct production patterns, in comparison to their production by peripheral cells. IFN-γ was produced in significant amounts in both stages whereas IL-17 was released only during the acute phase. Considering the local cytokine production, the most intriguing finding of our work was this apparent local disappearance of IL-17 producer cells during the chronic phase of the disease. This was considered a relevant finding because it has many implications in the actual status of knowledge in this area. Similar findings were reported by other authors. In acute EAE, a high number of CNS autoreactive Th17 cells are present in the inflamed CNS. High levels of CNS autoreactive Th17 cells are still present in the immune periphery but not in the CNS during EAE recovery period [28, 31]. This finding is also similar to reports made with CNS samples from MS patients. IL-17 and IFN-γ production by T cells has been associated with disease activity in MS patients and is also expressed in brain lesions. In addition, IL-17 expression in MS brain lesions [32, 33] and enrichment of IL-17-producing cells in glial cells, CD4+ and CD8+ T cells, were demonstrated by microarray analysis in the active rather than inactive areas of MS brain lesions [34]. Elevated frequencies of IL-17-producing cells have been associated with disease activity in the peripheral blood of MS patients [35, 36]. Interestingly, it has also been reported that although IL-17 and IFN-γ were elevated early during the disease, only IFN-γ enhancement was associated with relapse [37].

To try to understand the reduced local production of cytokines we checked the presence of T cells with presumed regulatory activity. Indeed, CD4+CD25+Foxp3+ T cells were found in the CNS, in both phases of the disease. Around 31% and 28% of the cells infiltrated in the brain, during the acute and chronic phases, respectively, were Foxp3+ regulatory T cells. These results are consistent with previous reports demonstrating the pivotal role of these cells in EAE control. It has been demonstrated that myelin-specific Treg cells are able to migrate and to accumulate in the CNS in animals with EAE [22, 27, 38]. In addition, higher frequency of Treg cells in the CNS have consistently been shown to correlate with recovery from EAE [22, 27]. These cells were not, however, sufficient to completely control the function of encephalitogenic T effector cells since the acute phase was followed by a chronic phase characterized by partial paralysis and persistent inflammation. These findings were very similar to the ones described by Korn et al., 2007 [22]. These authors clearly demonstrated that the expansion of Foxp3+ cells in the periphery was followed by their accumulation in the CNS. They also suggested that the inflammatory microenvironment was probably hindering the effective control of the autoimmune reaction by Foxp3+ cells.

Interestingly, this chronic inflammatory infiltrate was clearly distinct from the one observed during the acute disease phase. In this case, as described previously, there was still a local production of IFN-γ but not of IL-17. The mechanism of this change was not investigated. However, previous reports suggest that myeloid cells and the well-known plasticity of Th17 cells could be involved in this phenomenon. Myeloid effector populations present in the CNS include resident activated microglia and blood-derived monocytes, macrophages, and DCs [39]. These cells clearly mediate destruction of myelin sheets and axons in both MS and EAE [40]. However, convincing data indicate that they play a dual role. They initially promote T cell function by acting as APCs and also as effector cells activated by T lymphocytes. On the other hand, highly activated T cells trigger activation, including NO synthesis, of these myeloid

cells which, in turn, suppress ongoing T cell activity [41]. Myeloid cells as macrophages and DCs have been both involved in this contraction of the local immune response in EAE. Recently, in the EAE rat model, it was demonstrated that classically activated macrophages (M1) play a major pathogenetic role in disease initiation whereas alternatively activated macrophages (M2) contribute to disease recovery [42]. Procedures targeting the shift from M1 to M2 macrophages clearly reduced EAE severity in mice [43].

The contribution of myeloid-derived suppressor cells (MDSCs) to control EAE during the chronic phase is also supported by recent data. Moliné-Velázquez et al., 2011 [44], described that MDSCs limit neuroinflammation by promoting apoptosis of T lymphocytes in the spinal cord of mice with EAE. Ioannou et al., 2012 [45], demonstrated that granulocytic MDSCs accumulate within the CNS before EAE remission. Even more convincing was their observation that transfer of these cells was able to determine clinical improvement, decreased demyelination, and also inhibition of encephalytogenic Th1 and Th17 types of response.

Regarding Th17 plasticity, human and murine Th17 committed lymphocytes can turn to Th1 cells by upregulating T-bet and IFN-γ and downregulating IL-17 in the presence of Th1 polarizing factors [46, 47]. This *in vivo* plasticity was clearly demonstrated by Hirota et al, 2011 [48], by using an IL-17A reporter mouse. These authors showed that up to two-thirds of CNS-infiltrating Th17 cells, in mice with MOG-induced EAE, turned to Th1 cells by expressing their signature cytokine (IFN-γ). In this scenario we could think that the plasticity of Th17 cells is another factor that contributes to the observed absence of IL-17 during the chronic EAE phase.

Administration of IL-17 or adoptive transfer of Th17 myelin specific cells, during this chronic EAE stage, could certainly shed some light on this complex interplay of T cell subsets. This kind of analysis was not yet accomplished by us or other research groups. Even though this is a very unpredictable and complex subject, we are initially inclined to believe that both procedures would trigger similar outcomes; that is, they would exacerbate the disease. This prognosis is based on the literature data that shows improved clinical conditions upon IL-17 neutralization [28] and disease exacerbation after adoptive transfer of myelin-specific Th17 cells [49]. However, it is important to have in mind that Th17 transfer would include the contribution of the other cytokines produced by this Th subset [50]. In addition, Th17 cells would meet an inflammatory microenvironment in the brain that could affect their activity.

5. Conclusions

Together, these results demonstrated that chronic EAE phase, characterized by evident inflammatory infiltrates in the brain, is associated with persistent and high local IFN-γ production, absence of IL-17 synthesis, and local permanency of a high percentage of T CD4+CD25+Foxp3+ regulatory cells.

Conflict of Interests

All authors declare no conflict of interests.

Acknowledgments

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Research Article

The Causative Pathogen Determines the Inflammatory Profile in Cerebrospinal Fluid and Outcome in Patients with Bacterial Meningitis

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Background. The brain's inflammatory response to the infecting pathogen determines the outcome of bacterial meningitis (BM), for example, the associated mortality and the extent of brain injury. The inflammatory cascade is initiated by the presence of bacteria in the cerebrospinal fluid (CSF) activating resident immune cells and leading to the influx of blood derived leukocytes. To elucidate the pathomechanisms behind the observed difference in outcome between different pathogens, we compared the inflammatory profile in the CSF of patients with BM caused by *Streptococcus pneumonia* ($n = 14$), *Neisseria meningitidis* ($n = 22$), and *Haemophilus influenza* ($n = 9$). *Methods*. CSF inflammatory parameters, including cytokines and chemokines, MMP-9, and nitric oxide synthase activity, were assessed in a cohort of patients with BM from Burkina Faso. *Results*. Pneumococcal meningitis was associated with significantly higher CSF concentrations of IFN-γ, MCP-1, and the matrix-metalloproteinase (MMP-) 9. In patients with a fatal outcome, levels of TNF-α, IL-1β, IL-1RA, IL-6, and TGF-α were significantly higher. *Conclusion*. The signature of pro- and anti-inflammatory mediators and the intensity of inflammatory processes in CSF are determined by the bacterial pathogen causing bacterial meningitis with pneumococcal meningitis being associated with a higher case fatality rate than meningitis caused by *N. meningitidis* or *H. influenzae*.

1. Introduction

The three major pathogens causing bacterial meningitis (BM) are *Streptococcus pneumoniae* (*SP*), *Haemophilus influenzae type b* (*Hib*) and *Neisseria meningitidis* (*NM*). BM is the most severe and frequent infection of the central nervous system (CNS) and is associated with a high mortality rate and adverse neurological outcome in a substantial proportion of survivors [1]. BM caused by SP has the highest case fatality and neurological disability rates compared to those caused by NM or Hib [2, 3]. In a recent systematic review, the median inhospital case fatality ratio among African children with BM was 35% for SP, 25% for Hib, and 4% for NM meningitis [4]. In addition, about a quarter of children surviving pneumococcal meningitis and Hib meningitis had neuropsychological sequelae by the time of hospital discharge.

A number of factors have been identified as predictive for a poor outcome in terms of mortality. Coma and seizures were found to be predictive, next to shock, peripheral circulatory failure, severe respiratory distress, a low peripheral white blood cell (WBC) count, and a high CSF protein level in a recent systematic review of prognostic studies [5].

The host inflammatory reaction in the CNS is initiated by the recognition of the invading pathogens and results in the local production of soluble mediators. Differences in

the innate immune responses upon stimulation with Gram-positive and Gram-negative bacteria have been demonstrated in vitro and in experimental infection models. These differences are presumably related to pathogen-specific activation of pattern recognition receptors [6–9]. Brain cells, that is, astrocytes, microglial cells, endothelial cells, ependymal cells, and resident macrophages, react to the invading pathogens by releasing early response inflammatory cytokines, like IL-1β, TNF-α, and IL-6. TNF-α stimulates the recruitment of neutrophils and monocytes to the sites of infection and activates these cells to eliminate pathogens, by releasing reactive molecules, amongst others NO. Antibiotics causing rapid lysis of the bacteria have been shown to exacerbate CSF inflammation by increasing TNF-α [10]. After stimulation by bacterial wall components or TNF-α, IL-1β is released by mononuclear phagocytes, glial cells, and endothelial cells. High CSF level of IL-1β significantly correlates with adverse outcome and severity of BM [11]. Administration of TNF-α or IL-1β into the CSF results in pathophysiological changes characteristic of BM [12, 13]. IL-6 is produced by monocytes, endothelial cells, and astrocytes, mainly in response to IL-1β [14]. IL-10 and IL-1RA antagonize the effect of proinflammatory cytokines or chemokines, by inhibiting their production (IL-10) or acting as a decoy receptor (IL-1RA). CSF levels of other cytokines and chemokines (IL-2, IL-8, IFN-γ, MCP-1, MIP-1, and G-CSF) have also been found elevated in BM [15–19]. White blood cells invading the CSF release MMPs and reactive molecules [20–22] which are critically involved in the pathogenesis of brain damage in BM. Therapeutic strategies targeting MMPs and oxidative radical have yielded promising results, albeit limited to experimental BM models [20–24] to date. Tissue-destructive agents released by leukocytes and brain resident cells, like matrix-metalloproteinases (MMPs) and oxidants, also mediate brain damage in BM [22]. In BM, MMPs are involved in the blood-brain barrier opening, in immune cell extravasation, in the release of cytokines and cytokine receptors, and in the development of neuronal damage. In patients, elevated CSF levels of MMP-9 and MMP-8 have been detected [21], and high levels of MMP-9 were identified as a risk factor for sequelae [25]. Nitric oxide (NO) has been shown to contribute to the pathophysiology of meningitis with a phase-dependent role at the level of the cerebral vasculature by hyperemic effects in early phase and vasodilative effects protecting against ischemia in later phase [26].

Since all of the above-detailed inflammatory mediators have been shown to influence the outcome in experimental models of BM, we set out to determine in patients with BM the association between the CSF concentration of these mediators with the causative organism and the mortality. To this end the pathogen-specific inflammatory profiles caused by *S. pneumoniae*, *N. meningitidis*, and *H. influenzae* were analyzed in CSF from BM patients. The findings from this study may help understand, at a pathophysiological level, the difference in outcome observed between the different pathogens.

2. Materials and Methods

2.1. CSF Samples. CSF samples were collected in the Nouna Health District (NHD), Burkina Faso, during two consecutive meningitis seasons [27]. Ethical clearance for the meningitis study was obtained from the "Comite Local d'Ethique de Nouna" (Nouna Local Ethical Committee). Procedures followed were in accordance with the ethical standards of the committee and with the Helsinki Declaration of the World Medical Association. Informed consent was obtained from all study participants. Following the national guidelines for meningitis surveillance, diagnostic lumbar puncture was performed on patients with a suspicion of meningitis presenting to one of the 25 health centers of the NHD. Patients were enrolled into the study if their CSF could be transported on ice and analyzed by trained personnel in the laboratory of Nouna District Hospital within 6 hours. For primary analysis, white blood cell counts were determined. Samples were tested for bacterial pathogens using Gram staining, culture, latex agglutination, or PCR. CSF samples were centrifuged to remove white blood cells and supernatants stored at −80°C. Samples with conflicting diagnostics for the etiological agent between culture and PCR were excluded from the analysis. During transport from Africa to Switzerland, samples were kept frozen in liquid nitrogen.

CSF samples with confirmed acute BM (positive culture and/or positive PCR, CSF WBC of more than $50*10^6$ cells/liter) were categorized into three analytical groups according to the causative agent: *S. pneumoniae* (SP, $n = 14$), *N. meningitidis* (NM, $n = 22$), and *H. influenzae type b* (Hib, $n = 9$).

2.2. Assessment of Cytokine Levels in CSF Samples. Cytokine levels in CSF samples were assessed using microsphere-based multiplex assays (Lincoplex, LINCO Research Inc., St. Charles, MA, USA). CSF concentrations of the following cytokines were measured: IL-1α, IL-1β, IL-2, IL-6, IL-8, IL-10, IL-1RA, IFN-γ, MCP-1, MIP-1α, MIP-1β, TGF-α, and TNF-α. To fit the dynamic range of the test, samples were assessed undiluted or diluted 5- to 25-fold with the provided assay buffer, depending on the expected concentration of the respective analytes to be tested, as determined in preliminary experiments. A minimum of 50 beads per analyte was measured. Calibration curves from the provided standards were calculated using BioPlex Manager software version 4.1.1 with a five-parametric logistic curve fitting. When measured cytokine concentrations were below the detection limit, a value corresponding to the detection limit of the assay multiplied by the dilution factor of the sample was used for statistical analysis.

Validation of the assay was done for IL-10 and TNF-α using Enzyme-linked immunosorbent assays (ELISA) (R&D Systems Inc., Minneapolis, MN, USA). According to the concentrations estimated using the Lincoplex assay and the sensitivity range of the ELISA, samples were diluted 10-fold (TNF-α) or 20-fold (IL-10) to a final volume of 200 μL using the appropriate calibrator diluent. Results obtained by Luminex and conventional ELISA were compared for correlation, using Prism Software. For both cytokines, a significant correlation was found between the two methods (TNF-α, $P = 0.002$, Spearman $r = 0.53$; IL-10, $P < 0.0001$, Spearman $r = 0.75$).

2.3. Assessment of MMP-9 Levels in CSF Samples. MMP-9 levels were assessed using the Fluorokine MAP Human MMP Kit (R&D Systems Inc., Minneapolis, MN, USA). All CSF samples were diluted 100-fold, to a final volume of 50 μL. A minimum of 50 beads was measured. Standard curves were calculated similarly to those of the cytokines assay.

Validation of the assay was done using gelatin-containing gel zymography as already described [21]. Concentrations measured by the Fluorokine MAP assay correlated with those assessed by gelatin zymography with a two-tailed P-value of <0.0001 (Spearman's rank correlation test, $r = 0.66$).

2.4. Measurement of Total Nitrate and Nitrite in CSF Samples. CSF levels of total nitrate and nitrite were assessed using a nitrate/nitrite colorimetric assay (Cayman Chemical Company, Ann Arbor, MI, USA). The estimated concentrations were used as an index for nitric oxide synthase activity. CSF samples were filtered for 30 min at 10000 g using Ultrafree −0.5 centrifugal filter devices. Samples and assay buffer (each 40 μL) were mixed with 10 μL of coenzyme mixture and 10 μL of nitrate reductase in a 96-well plate. After 3 h at room temperature (RT) for conversion of nitrate to nitrite, Griess reagents were added for 10 min at RT. Absorbance was measured at 550 nm. Total nitrite concentrations were calculated using standard curves generated by the SoftMax PRO software version 3.1.2 (Molecular Devices Inc., Sunnyvale, CA, USA) using a linear curve fitting.

2.5. Statistical Analysis. Statistical analysis was done using GraphPad Prism version 5.04 (GraphPad Software Inc., La Jolla, CA, USA). For comparison of the different pathogen groups, we first tested whether data sets followed a Gaussian distribution. At least one group did not follow a Gaussian distribution for each comparison. Furthermore, since we had to include arbitrary values, the nonparametric Kruskal-Wallis test was used. If the overall test was significant ($P < 0.05$), the Mann-Whitney test was applied to perform pairwise comparisons. For the analysis of the relation between CSF cytokine levels and the outcome of the disease, the nonparametric Mann-Whitney test was used, with a confidence interval of 95% and two-tailed P values. Correlations were analyzed using Spearman's rank correlation test, with a confidence interval of 95% and two-tailed P values.

3. Results

3.1. Clinical Parameters. Significant pathogen-specific differences in the age distribution of patients were observed within the study cohort (P Kruskal-Wallis test: $P < 0.01$). NM meningitis was found in patients 0–60 years (median: 5.5 years, $n = 22$) and SP meningitis in patients 0–40 years (median: 5.5 years, $n = 14$). In contrast, Hib meningitis affected only children 1–4 years (median: 2 years, $n = 9$). The difference in median ages was significant between SP versus Hib ($P < 0.05$) and NM versus Hib ($P < 0.01$). Mortality of BM patients was 46% for SP and 27% for NM, while all nine patients infected with Hib survived (Table 1).

CSF white blood cell counts did not significantly differ within the 3 groups (median SP: 7020×10^6/L [100–64000]; median NM: 4900×10^6/L [100–38560]; median Hib: 5540×10^6/L [272–20000]) (Table 2).

3.2. Cytokine and Chemokine Levels in CSF. Cytokines and chemokines showed significantly different regulations between the causative bacteria (Table 2 and Figure 1). In particular, the CSF concentration of IFN-γ was significantly higher in patients infected with SP compared to NM ($P < 0.005$) and Hib ($P < 0.005$). The CSF concentration of IFN-γ correlated with the age of the patients ($P = 0.15$, Pearson's $r = 0.38$; Figure 2). This correlation was significant for meningitis caused by SP ($P = 0.01$, $r = 0.79$) and NM ($P = 0.02$, $r = 0.64$). This suggests that, during meningitis, adults are more apt to react with IFN-γ production than children [28]. Since there was a statistically significant difference in age for the Hib group, we cannot exclude that the difference in IFN-γ level in this population may be due to the age, rather than the pathogen. For MCP-1 significant differences between SP versus NM patients ($P = 0.045$) and SP versus Hib patients ($P < 0.01$) were observed (Table 2). In addition, a nonsignificant trend for SP causing higher levels of IL-1β ($P < 0.07$) and IL-6 ($P = 0.055$) was found. Taken together, reciprocal trends in the association of pro- and anti-inflammatory cytokines and chemokines with BM caused by the different pathogens were observed. Il-1β, IFN-γ, and MCP-1, as prototypical proinflammatory factors, showed higher CSF concentrations in the patients infected with SP than by NM and Hib. In contrast, the anti-inflammatory mediators IL-10 and IL-1RA, were more increased in CSF of patients infected with NM and Hib. The ratio of pro- to anti-inflammatory mediators, in particular the IL-1β/IL-1RA ratio, showed statistically significant differences, being higher in SP versus NM ($P < 0.01$) and SP versus Hib ($P < 0.03$). Similar correlations were found for IL-6/IL-10 and IL-6/IL-1RA ratios (Figure 3). Cyto-/chemokines concentrations were significantly higher in patients infected with any of the 3 pathogens when compared with a group of 7 healthy control patients, as defined by no clinical signs of meningitis and no increase in WBC in the CSF (median 4×10^6 cells/L). For IL-1β, IL-2, TNFα, IFNγ, and MIP1 α, the majority of these samples were under detection limit, even when samples were analyzed undiluted (Table 3).

Since the host inflammatory reaction during BM is an important determinant of disease severity and mortality, the association between CSF cytokine and chemokine levels and outcome (survival or death) was investigated (Table 4(a) and Figure 4). When all patients were analyzed together, a significant association between fatal outcome and CSF levels of 5 cytokines, namely, IL-1β, TNF-α, IL-1RA, IL-6 ($P < 0.05$), and TGF-α ($P < 0.02$), was found. When pathogens were investigated separately, TNF-α was significantly higher in patients who died from SP meningitis (Table 4(b)), while only IL-1RA was significantly higher in patients with a fatal outcome after NM meningitis (Table 4(c)).

3.3. MMP-9, Nitrate and Nitrite Levels in CSF. In children with bacterial meningitis, matrix-metalloproteinase-(MMP-) 9 in the cerebrospinal fluid has been associated with

Table 1: Patient groups characteristics.

Pathogen	Number of patients	Gender (male/female)	Median age (min–max)	Mortality (%) (survivors/death)
S. pneumoniae	14	5/8[1]	5.5 (0–40)	46% (7/6)[1]
N. meningitidis	22	10/9[2]	5.5 (1–60)	27% (16/6)
H. influenzae	9	7/2	2 (1–4)	0% (9/0)

[1]1 patient not documented.
[2]3 patients not documented.

Table 2: CSF inflammatory parameters of patients infected with SP ($n = 14$), NM ($n = 22$), and Hib ($n = 9$).

	Streptococcus pneumoniae Median (min.–max.) (pg/mL)	*Neisseria meningitidis* Median (min.–max.) (pg/mL)	*Haemophilus influenzae* Median (min.–max.) (pg/mL)	*P* Kruskal-Wallis	Between group significance
IL-1β	116.2 (1.79–1040)	23.01 (0.61–325.9)	13.3 (0.77–112.5)	0.069	(b)
IL-2	3.28 (0.38–19.81)	1.41 (0.38–25.69)	1.6 (0.18–3.83)	ns	
IL-6	106232 (4440–175553)	93316 (5917–162002)	23257 (3917–112594)	0.0551	(b)
IL-10	7515 (509.5–44609)	18604 (1883–217931)	12692 (1466–135980)	ns	
IL-1RA	103123 (4413–983243)	243089 (3183–639929)	216530 (7712–1301000)	ns	
TNF-α	233.8 (34.85–1199)	318.2 (17.6–3390)	170.3 (43.63–1395)	ns	
IFN-γ	58.99 (8.03–2853)	8.94 (1.77–219.8)	8.62 (0.42–20.03)	<0.01	(a), (b)
MCP-1	10109 (1691–23567)	1896 (309.8–40005)	2059 (411.4–3897)	<0.04	(a), (b)
MIP-1α	829.1 (30.75–3828)	1469 (133.8–27027)	1152 (135.6–12782)	ns	
MIP-1β	3344 (575.8–7518)	3263 (530.7–90852)	2563 (1364–22695)	ns	
TGF-α	73.4 (16.36–302.3)	65.26 (10.3–146.5)	43.23 (27.52–305.2)	ns	
MMP-9	1.51×10^6 ($275632–4.5 \times 10^6$)	525821 ($1877–5.32 \times 10^6$)	334058 ($12440–2.006 \times 10^6$)	<0.03	(a), (b)
WBC	7020 (100–64000)	4900 (100–38560)	5540 (272–20000)	ns	
Nitrite/nitrate*	19.8 (9.5–104)	37 (19.24–124.1)	21.36 (4.533–41.5)	0.059	(c)

The nitrite/nitrate concentration (NO) was determined in a subset of CSFs, due to limitations in the available sample volumes (Hib $n = 8$, SP = 8, and NM = 12). The column entitled "Between group significance" describes statistical significance between groups as determined by Mann-Whitney test, for the following comparisons (a) SP versus NM, (b) SP versus Hib, and (c) NM versus Hib.

Table 3: CSF inflammatory parameters in control patients ($n = 7$).

Analytes	Samples under detection limit	Median (pg/mL) [min.–max.]	Dilution factor	Detection limit
IL-1β	5/7	n.d	1 : 1	0.19
IL-2	7/7	n.d.	1 : 1	0.38
IL-6	0/7	115 [95.06–175.1]	1 : 1	0.79
IL-10	1/7	39.96 [0.41–291.3]	1 : 1	0.41
IL-1RA	0/7	184.6 [99.82–2958]	1 : 1	10.76
TNF-α	4/7	n.d	1 : 1	0.22
IFN-γ	7/7	n.d	1 : 1	0.55
MCP-1	0/7	326 [116–11355]	1 : 5	0.63
MIP-1α	2/7	35.43 [1.23–536.6]	1 : 1	1.23
MIP-1β	4/7	n.d	1 : 1	27.65
TGF-α	0/7	24.16 [13.77–30.3]	1 : 1	0.69
MMP-9	0/7	106.8 [10.93–1153]	1 : 10	n.d.

A median value was calculated only when the majority of samples were above the limit of detection. Control samples were measured undiluted or diluted 1 : 5, respectively, 1 : 10.
[1]Detection limit as provided by the manufacturer.

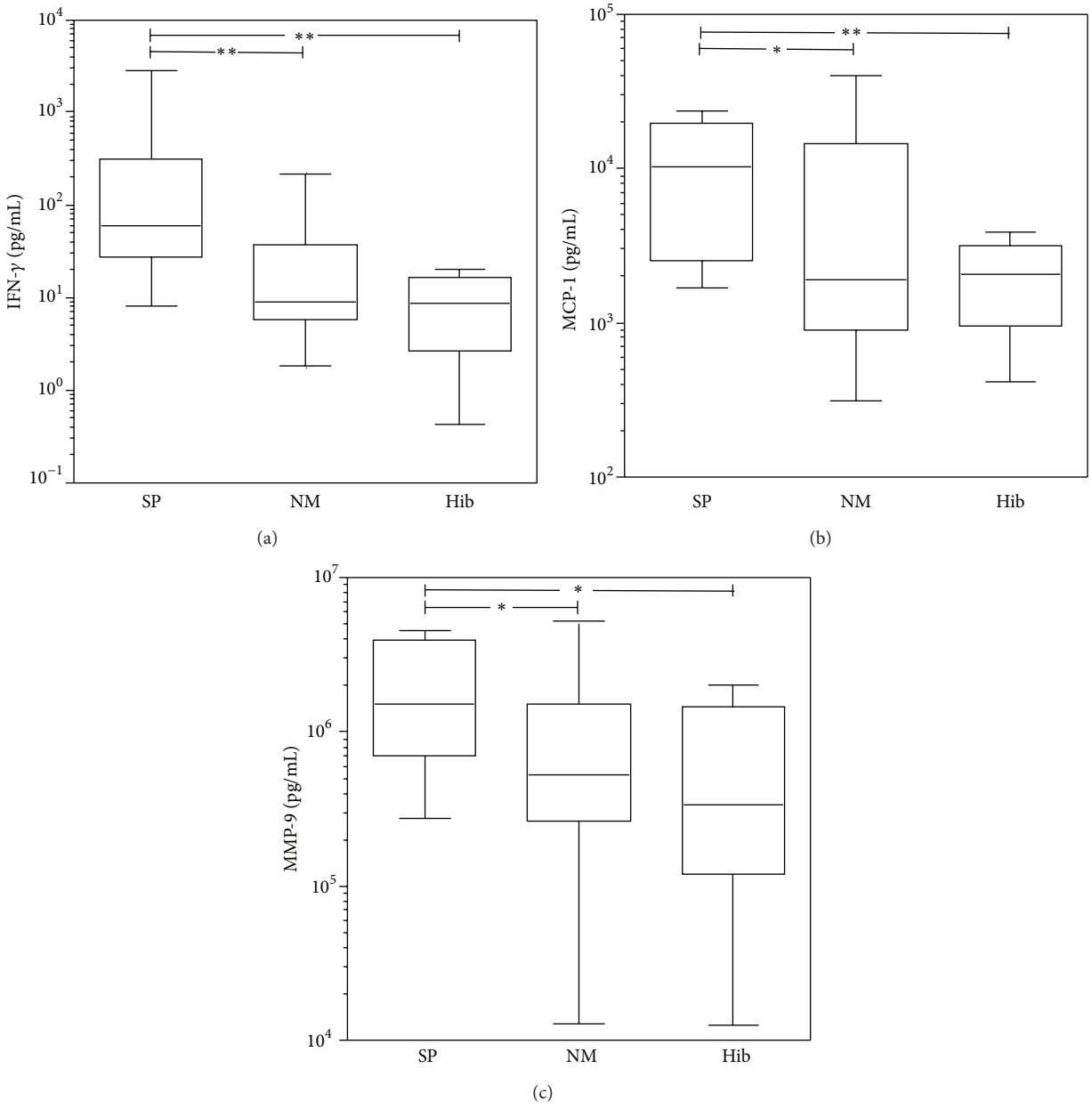

Figure 1: Inflammatory CSF parameters in BM patients. Statistically significant differences ($^*P < 0.05$; $^{**}P < 0.01$) in CSF concentrations of MCP-1, IFN-γ, and MMP-9 were observed in patients with BM grouped for the causative pathogens.

blood-brain barrier damage and neurological sequelae [25]. Concentrations of MMP-9 were highest in CSF of patients suffering from SP meningitis ($P < 0.03$; Table 1 and Figure 1). Pairwise comparisons between the different etiological agents revealed SP versus Hib to differ significantly ($P < 0.02$) as well as SP versus NM ($P < 0.04$) (Figure 1). MMP-9 showed a nonsignificant trend towards higher CSF levels in patients who died from the disease (Mann-Whitney test: $P = 0.068$, Table 4(a)).

Levels of total nitrate and nitrite showed a nonsignificant trend ($P \leq 0.06$) between pathogens, with higher CSF concentrations in samples of NM patients than in SP and Hib patients (Table 1).

4. Discussion

In addition to the high mortality of up to 30%, cases of BM and specifically those caused by SP are associated with persistent neurological sequelae in up to 50% of the survivors due to different forms of brain damage [29, 30]. The burden of disease is especially high in low-income countries, and risk of mortality or major sequelae is twice as high in African as

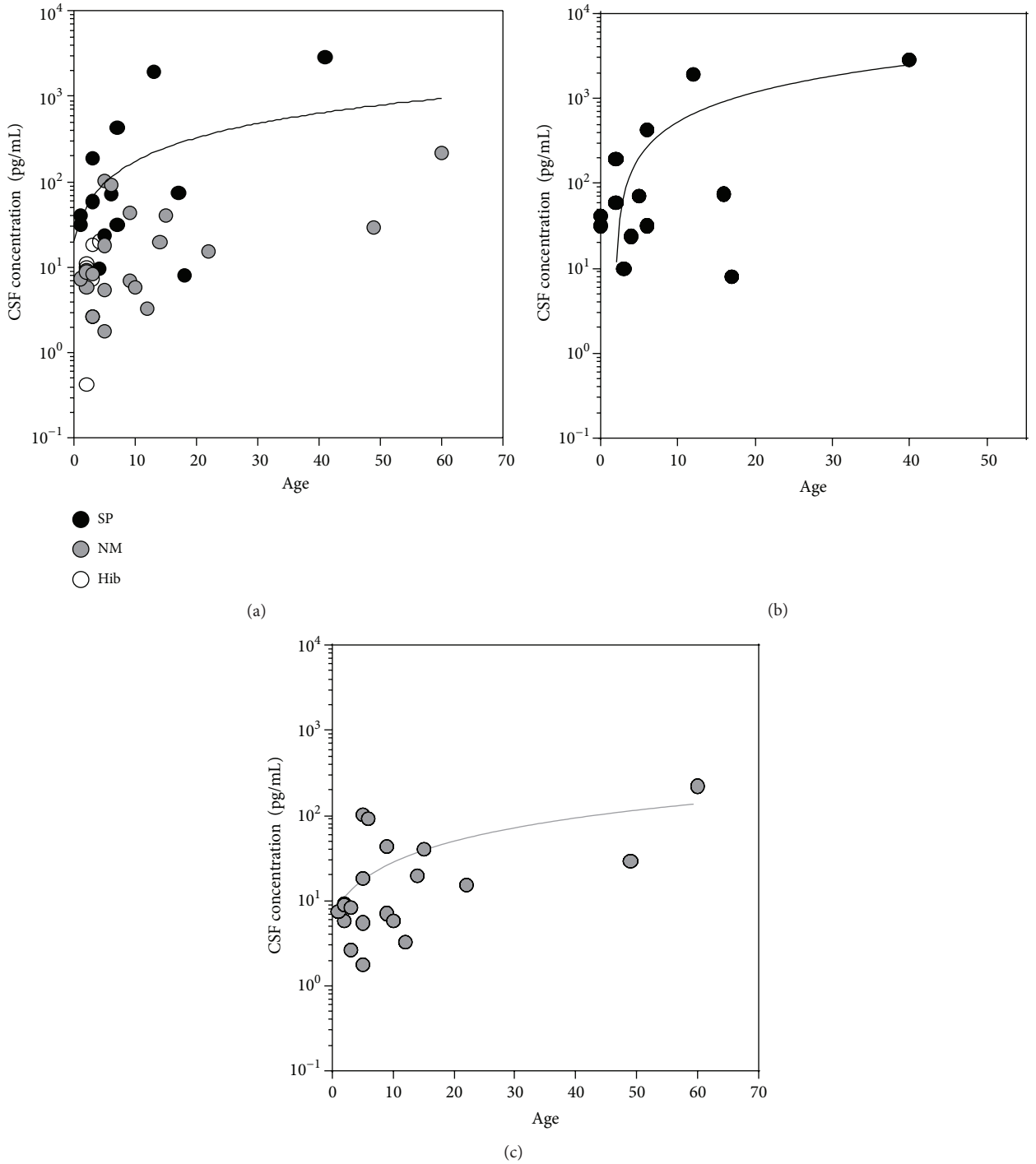

Figure 2: Correlation between CSF levels of IFN-γ and age of the patients. (a) The level of IFN-γ correlated with the age of the patients. This correlation was significant for SP patients ((b): $P = 0.01$, $r = 0.79$, and black dots) and NM patients ((c): $P = 0.02$, $r = 0.64$, and grey dots).

in the European regions [31]. Over the last four decades, the risk of major postdischarge sequelae caused by meningitis has not significantly changed [31]. Both clinical and experimental studies suggest that both the pathogen and the inflammatory host response contribute to the development of mortality and neurological sequelae.

Here we compared the host immune response in the CSF to BM caused by *S. pneumoniae*, *N. meningitidis*, and *H. influenzae*. To date, only few studies have compared the CSF concentration of inflammatory mediators during BM in relation to the bacterial pathogen [17, 28, 32]. Here we found that the pathogen is an important determinant of

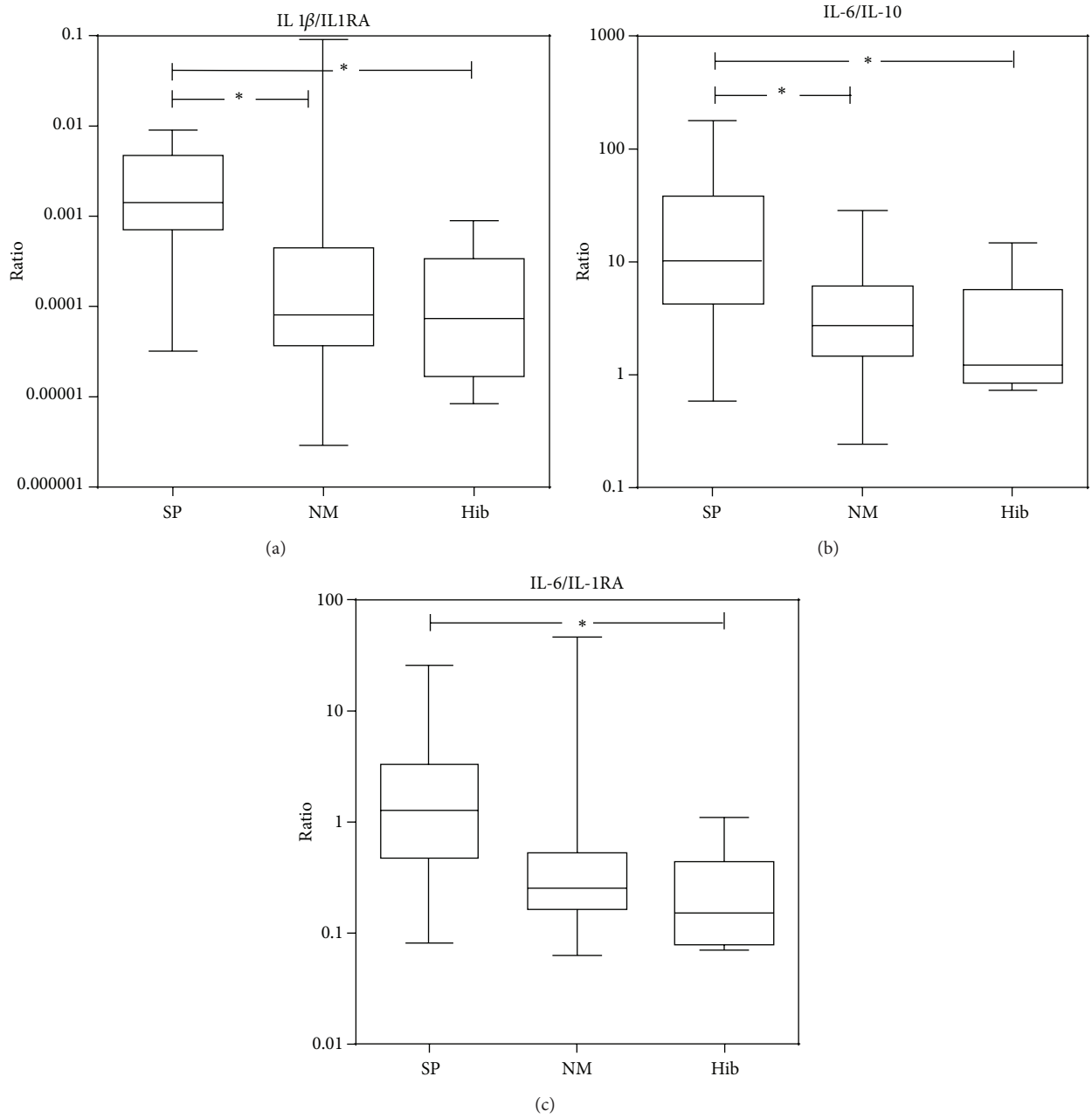

Figure 3: Ratio of pro- to anti-inflammatory mediators. Statistically significant differences ($^*P < 0.05$) in the ratios of IL-1β/IL-1RA, IL-6/IL-10, and IL-6/IL-1RA were observed in patients with BM grouped for the causative pathogens.

the inflammatory CSF reaction to BM. The observed difference in inflammation in the CSF may not only be due to inherent differences between pathogens to elicit a response in cells of the innate immune system [6–9] but also due to the ability of the pathogen to multiply in the CNS compartment. Unfortunately, determining the bacterial load in the CSF of patients was not feasible in the present study.

In accordance with published data [17, 28], we observed significantly higher CSF concentration of IFN-γ in pneumococcal meningitis. Furthermore, as observed by others, the level of IFN-γ correlated with the age of the patients. This suggests that, during meningitis, adults are more apt to react with IFN-γ production than children [28]. IFN-γ is a potent proinflammatory cytokine. It enhances the function of macrophages and polymorphonuclear leukocytes by stimulating nonspecific defense mechanisms such as phagocytosis and the release of inflammatory mediators and may therefore contribute to the overshooting inflammation.

We found elevated levels of the chemokines MCP-1, MIP-1α, and MIP-1β in the CSF of patients with BM, in accordance with other published studies [18, 19, 33]. CSF concentrations of MCP-1 were significantly higher in patients infected with

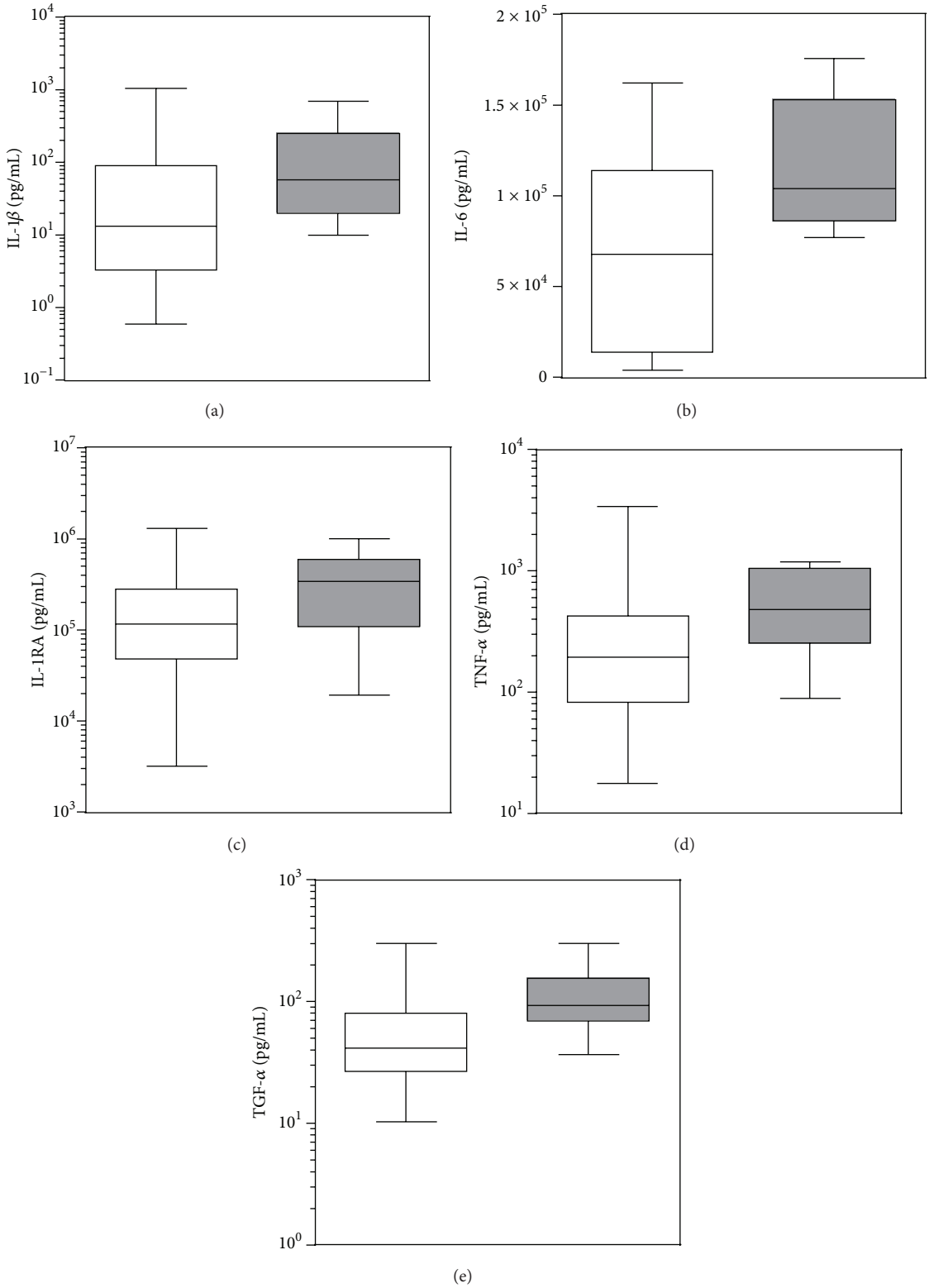

Figure 4: Significant differences in cytokines CSF levels in relation to disease outcome. Patients were grouped independently of the etiological agents based on the outcome (nonlethal, white boxes/lethal, grey boxes) of the disease. Pairwise comparisons (Mann-Whitney test) revealed statistically significant differences for the 5 cytokines (see also Table 4).

Table 4: Difference in CSF inflammatory parameters between patients with a nonfatal and a fatal outcome.

(a) Pooled pathogens

	Nonfatal cases Median (min.–max.) (pg/mL)	Fatal cases Median (min.–max.) (pg/mL)	Mann-Whitney
IL-1β	*13.19 (0.61–1040)*	*57.19 (9.84–682)*	*0.046*
IL-2	1.6 (0.18–255.69)	1.41 (0.38–6.9)	0.6618
IL-6	*67789 (3917–162002)*	*104035 (77100–175553)*	*0.0365*
IL-10	15764 (509.5–217931)	14131 (809–90755)	0.9351s
IL-1RA	*117918 (3183–1.301 × 10^6)*	*337610 (19362–983243)*	*0.0209*
TNF-α	*195.1 (17.6–3390)*	*484.3 (88.41–1199)*	*0.0436*
IFN-γ	11.16 (0.42–1920)	49.13 (1.77–2853)	0.1823
MCP-1	2101 (309.8–40005)	2903 (1187–20728)	0.2193
MIP-1α	935.6 (133.8–27027)	1166 (30.75–16122)	0.9351
MIP-1β	2728 (530.7–24661)	3555 (1263–90852)	0.4177
TGF-α	*41.05 (10.30–305.2)*	*93.3 (36.74–302.3)*	*0.0099*
MMP-9	463489 (1877–5.319 × 10^6)	1.258 × 10^6 (313541–4.497 × 10^6)	0.0671
WBC	4600 (100–186000)	11100 (1000–42400)	0.062
Nitrite/nitrate*	137.5 (23.15–625)	226.9 (83.6–368.5)	0.3627

(b) SP only

	Non-fatal cases (n = 7) Median (min.–max.) (pg/mL)	Fatal cases (n = 5) Median (min.–max.) (pg/mL)	Mann-Whitney
IL-1β	54.44 (1.79–1040)	206.9 (9.84–682)	0.5253
IL-2	7.87 (0.38–19.81)	1.6 (0.38–5.55)	0.1452
IL-6	106232 (4440–150456)	136215 (77100–175553)	0.3434
IL-10	7515 (509.5–44609)	7497 (809–17392)	0.6010
IL-1RA	55240 (4413–275000)	121576 (19362–983243)	0.1591
TNF-α	*70.65 (0.22–570.9)*	*416.2 (88.41–1199)*	*0.048*
IFN-γ	40.82 (8.03–1920)	190.7 (23.73–2853)	0.202
MCP-1	10109 (1691–23059)	2897 (2020–20307)	0.8207
MIP-1α	829.1 (256.9–3828)	416.4 (30.75–1738)	0.5025
MIP-1β	2732 (575.8–6685)	3771 (1263–7342)	0.6313
TGF-α	35.52 (16.36–153.5)	100.5 (36.74–302.3)	0.149
MMP-9	1.103 × 10^6 (275632–4.254 × 10^6)	2.676 × 10^6 (475112–4.497 × 10^6)	0.2331
WBC	4600 (100–64000)	12930 (3200–42400)	0.1375

(c) NM only

	Non-fatal cases (n = 15) Median (min.–max.) (pg/mL)	Fatal cases (n = 5) Median (min.–max.) (pg/mL)	Mann-Whitney
IL-1β	10.56 (0.61–325.9)	50.5 (10.48–205.4)	0.3056
IL-2	1.41 (0.38–25.69)	1.22 (0.38–6.9)	0.916
IL-6	67789 (5917–162002)	1100160 (77742–145357)	0.3056
IL-10	16715 (1883–217931)	47476 (5436–90755)	0.1974
IL-1RA	*182057 (3183–305690)*	*447933 (275000–639929)*	*0.001*
TNF-α	267.2 (17.6–3390)	552.4 (247.4–1126)	0.2661
IFN-γ	9.1 (2.62–219.8)	8.3 (1.77–102.7)	0.5413
MCP-1	1283 (309.8–40005)	2909 (1187–20728)	0.3056
MIP-1α	1200 (133.8–27027)	1937 (585.1–16122)	0.444
MIP-1β	3137 (530.7–24661)	3388 (1851–90852)	0.5528
TGF-α	44.05 (10.3–146.5)	86.05 (58.93–144.7)	0.0526
MMP-9	585934 (1877–5.319 × 10^6)	525821 (313541–2.188 × 10^6)	0.7996
WBC	4900 (100–17000)	7800 (1000–38560)	0.3983

Significantly higher CSF concentrations in patients with a fatal outcome are represented in italic lines.

SP compared to both NM and Hib. Elevated CSF levels of MCP-1 have been shown to exacerbate brain damage during neuroinflammatory diseases by increasing the influx of monocytes and neutrophils [34]. However, we did not find a correlation of MCP-1 levels with WBCs in the CSF, which is in accordance with other studies [18, 33]. Furthermore, WBC count did not correlate with any other parameters, either when pathogen groups were pooled together or when analyzed separately. Worsening of the outcome by an overshooting inflammatory reaction is further suggested by the increased ratios of pro- to anti-inflammatory mediators (IL-6/IL-10, IL6/IL-1RA, and IL-1β/IL-1RA) observed in patients infected by SP. In a previous study in BM patients, CSF showed higher ratio of TNF-α/IL-10 by SP when compared to NM and Hib combined [17].

High CSF concentration of MMP-9 is a risk factor for a detrimental outcome [25, 35, 36]. Our results add further support to the notion that MMP-9 is critically involved in the increase in mortality and sequelae, since CSF levels of MMP-9 were significantly higher in BM caused by SP, which is usually associated with a higher incidence of neurological sequelae and mortality [25].

When the relationship between inflammatory mediators and the outcome of the disease was investigated independently of the causative agent, higher CSF levels of TNF-α, IL-1β, IL-1RA, IL-6, and TGF-α were found in patients who died from BM. A nonsignificant trend was also found for MMP-9 and IL-6. Thus, the present study confirms previous observations showing that both a strong activation of the IL-1β system [37] and increased MMP-9 levels [25] correlate with adverse outcome of BM. While the higher mortality observed in SP may be seen in the context of higher cytokine levels, this observation could not be made for the difference in mortality between NM (27%) and Hib (0%) where inflammatory CSF parameters were not significantly different. In comparison to other studies in which mortality in developing regions reached 30% [38], the mortality attributed to Hib was exceptionally low in the present study. The small group size for Hib is a clear limitation of the study. Furthermore, NM meningitis is more often associated with fulminant septicemia, which may contribute to mortality. Unfortunately, data concerning the presence of concomitant septicemia were not available in the present study. Interestingly, the inhibition of the metalloproteinase TACE/ADAM17, acting as a sheddase for TNF-α and TGF-α [39], has been shown to lower mortality and to attenuate brain injury in experimental models of BM [20, 36]. In line with these results, we could show in the present study that TNF-α was significantly upregulated in patients with a poor outcome. In accordance with this clinical observation is the experimental finding that deletion of another member of the TGF family, TGF-β has been shown to improve bacterial clearance and diminished intracranial complication in a mouse with pneumococcal meningitis [40].

A nonsignificant trend for higher levels of nitrite/nitrate levels was observed in NM infected patients. In experimental models, CSF NO/nitrite concentration correlated with an increase in blood-brain barrier permeability, but inhibition of the different nitric oxide synthases resulted in inconsistent effects, probably as a result of differences in the timing of intervention and the corresponding effects on the brain perfusion.

The present study, analyzing a cohort of patients affected by meningitis, identified several factors which contribute to the worsening of outcome in bacterial meningitis. Interestingly, some of these factors (TNF-α, MMPs, and nitric oxide) have already been described in experimental models using knockout animals [41] and/or intervention strategies [42] which reduced mortality and ameliorated the outcome of infected animals. The most promising strategies derived from these experimental models include the reduction of the inflammatory reaction by targeting different steps in the inflammatory process [43], from the release of proinflammatory bacterial products to the activation of the innate immune system and the production/release of cytokines or chemokines, as well as the inhibition of metalloproteinases or treatments with antioxidants [44].

5. Conclusion

In conclusion, this study showed that SP, NM, and Hib elicit distinct profiles of inflammatory mediators in the CSF during BM. A more intense inflammatory reaction, in particular higher CSF levels of IFN-γ, MCP-1, and MMP-9, were observed in patients infected with SP. Furthermore, the ratios of pro- to anti-inflammatory parameters were found to be significantly higher in patients with SP meningitis. This is likely to contribute to the higher case fatality rate and morbidity observed in patients suffering from pneumococcal meningitis and may therefore help find new treatment strategies aimed at improving the outcome of infected patients.

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Research Article

MMP-3 Contributes to Nigrostriatal Dopaminergic Neuronal Loss, BBB Damage, and Neuroinflammation in an MPTP Mouse Model of Parkinson's Disease

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The present study examined whether matrix metalloproteinase-3 (MMP-3) participates in the loss of dopaminergic (DA) neurons in the nigrostriatal pathway in a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of Parkinson's disease with blood brain barrier (BBB) damage and infiltration of peripheral immune cells. Tyrosine hydroxylase (TH) immunostaining of brain sections from MPTP-treated mice showed that MPTP induced significant degeneration of nigrostriatal DA neurons. Moreover, FITC-labeled albumin detection and immunostaining revealed that MPTP caused damage to the BBB and increased the number of ED-1- and CD-3-immunopositive cells in the substantia nigra (SN). Genetic ablation of MMP-3 reduced the nigrostriatal DA neuron loss and improved motor function. This neuroprotective effect afforded by MMP-3 deletion was associated with the suppression of BBB disruption and a decrease in the number of ED-1- and CD-3-immunopositive cells in the SN. These data suggest that MMP-3 could play a crucial role in neurodegenerative diseases such as PD in which BBB damage and neuroinflammation are implicated.

1. Introduction

Parkinson's disease (PD) is a common neurodegenerative disease associated with progressive degeneration of the nigrostriatal dopaminergic (DA) pathway [1]. Although the etiology of PD and the mechanisms that mediate disease development remain largely unknown, accumulating clinical and experimental evidence suggests that PD is associated with neuroinflammatory processes such as microglial activation, T-leukocyte infiltration, and blood brain barrier (BBB) dysfunction [2–4]. Microglial and/or macrophage phagocytotic activity [3] and T-leukocyte infiltration [5] are upregulated in damaged areas of the midbrain of PD patients and in the brains of MPTP-treated mice, thus giving rise to the death of DA neurons. Increased BBB permeability [6] and blood vessel changes [7] have also been reported in PD patients and similarly contribute to DA neuronal death in MPTP-treated animal models of PD [8, 9].

Matrix metalloproteinase-3 (MMP-3) is a zinc-dependent proteolytic enzyme that is converted to active MMP-3 through autocleavage; the active form remodels the extracellular matrix complex (EMC) in the basal lamina which forms part of the BBB [10]. Besides degradation of ECM molecules, MMP-3 can activate pro-MMPs (pro-MMP-1, -3, -7, -8, -9, and -13) and cleave cell adhesion molecules, chemokines, and cytokines [11]. The widespread distribution of MMP-3 in the brain suggests that it plays a crucial role in the central nervous system (CNS). MMP-3 is involved in axonal growth,

neuronal migration and synaptogenesis in brain development [12, 13], and in synaptic plasticity in learning and memory [14]. In contrast to these functions, it has been shown that MMP-3 released from apoptotic neuronal cells causes microglia activation and increases inflammatory processes *in vitro* [15]. In addition, MMP-3 facilitates BBB disruption and neutrophil influx in the cerebral cortex of LPS-injected mice as a consequence of its action on the basal lamina and tight junction proteins [16].

Increasing evidence suggests that MMP-3 plays an important role in the pathogenesis of neurodegenerative diseases such as Alzheimer's disease, vascular dementia, ischemic stroke, and PD [10]. In particular, several *in vitro* studies reported that MMP-3 exerted neurotoxic effects on oxidative stress- and endoplasmic reticulum stress-triggered DA neuronal death through caspase-3 activation [17, 18]. Moreover, MMP-3 immunoreactivity was elevated in the SN of 6-hydroxydopamine-(6-OHDA-) injected rats [19] and colocalized within Lewy bodies in the postmortem brains of PD patients [20]. Under the neuropathological conditions of PD, MMP-3 participates in DA neuronal cell death through the proteolytic cleavage of α-synuclein *in vivo* and *in vitro* [20]. In addition, ghrelin and exendin-4 have been shown to attenuate nigrostriatal DA neuronal loss and microglial activation via inhibition of MMP-3 expression in the MPTP mouse model of PD [21, 22]. However, in the context of PD, little is known about the role of MMP-3 in relation to BBB function and the infiltration of peripheral immune cells in the nigrostriatal DA system. In the current study, therefore, we have used the MPTP mouse model of PD with a view to determining if MMP-3 exacerbates the degeneration of nigrostriatal DA neurons as a consequence of its action to disrupt the BBB and allow the infiltration of T leukocytes into the brain.

2. Materials and Methods

2.1. Animals and Treatment. Eight-to nine-week-old male C57BL/6J mice ($MMP^{+/+}$; Jackson Laboratory) and C57BL/6J-inbred mice deficient for matrix metalloproteinase-e ($MMP\text{-}3^{-/-}$) were used [23]. All experiments were performed in accordance with the approved animal protocols and guidelines established by Kyung Hee University (KHUASP(SE)-10-030). For MPTP intoxication, mice received four intraperitoneal injections of MPTP (20 mg/kg, free base; Sigma) dissolved in saline at 2 h intervals by following the previously reported method [24, 25].

2.2. Tissue Preparation and Immunohistochemistry. Mice brain tissues were prepared for immunostaining as described previously [24–26]. Briefly, brain sections were rinsed in PBS and incubated overnight at room temperature with primary antibodies. The primary antibodies included those directed against tyrosine hydroxylase (TH, 1 : 2000 dilution; Pel-Freez Biologicals, Rogers, AR, USA) for DA neurons, ED-1 (CD68, 1 : 1000 dilution; Serotec, Oxford, UK) for phagocytotic microglia/macrophage, and CD-3 (1 : 500 dilution; Serotec, Oxford, UK) for T leukocytes. The following day, sections were rinsed with PBS and 0.5% bovine serum albumin (BSA), incubated with the appropriate biotinylated secondary antibody, and processed with an Avidin-Biotin Complex Kit (Vectastain ABC kit; Vector Laboratories, Burlingame, CA, USA). Bound antiserum was visualized by treatment with 0.05% diaminobenzidine-HCl (DAB) and 0.003% hydrogen peroxide in 0.1 M PB. The DAB reaction was terminated by rinsing tissues in 0.1 M PB. Labeled tissue sections were mounted on gelatin-coated slides and analyzed under a bright-field microscope (Nikon, Melville, NY, USA).

2.3. Stereological Cell Counts. The unbiased stereological estimation of the total number of the TH-ip neurons was made using the optical fractionator method performed on an Olympus Computer-Assisted Stereological Toolbox (CAST) system version 2.1.4 (Olympus Denmark A/S, Ballerup, Denmark) in the various animal groups at 7 days postinjection (MPTP or saline) as previously described [25, 27]. Actual counting was performed using a 100x oil objective. The total number of neurons was estimated according to the Optical Fractionator Equation [28]. More than 300 points over all sections of each specimen were analyzed.

2.4. Densitometric Analysis. As previously described [25, 27], the optical density of TH-positive fiber in STR was examined at 5x magnification using the Image Pro Plus system (Version 4.0, Media Cybernetics, Silver Spring, Maryland, USA) on a computer attached to a light microscope (Zeiss Axioskop, Oberkochen, Germany) interfaced with a CCD video camera (Kodak Mega Plus model 1.4 I, New York, NY, USA). To determine the density of the TH staining in the STR, a square frame of 700 × 700 mm was placed in the dorsal part of the STR. A second square frame of 200 × 200 mm was placed in the region of the corpus callosum to measure background values. To control variations in background illumination, the average of background density readings from the corpus callosum was subtracted from that of density readings of the striatum for each section. For each animal, the average of all sections was calculated separately before data were statistically processed.

2.5. Rotarod Test. We measured the ability of the animal to balance itself and remain on an accelerating rotarod (UgoBasile, Comerio, Italy) using two different experimental designs (fixed mode and accelerating mode) as previously described [25, 29]. To acclimate mice on the rotarod apparatus, animals were given a training session (10 rpm for 20 min), 7 consecutive days before MPTP injection. Animals that stayed on the rod without falling during training were selected and randomly divided into experimental groups. Seven days after the last MPTP treatment, animals receiving various treatment regimes were placed on the rotating rod and tested at 20 rpm for 20 min as fixed mode. On the next day, mice were tested at different progressively higher speeds on the rotarod apparatus that accelerates at a constant rate, from 4 to 40 rpm in 300 s as accelerating mode. The latency to fall was automatically recorded by magnetic trip plates.

2.6. FITC-Labeled Albumin Assay. As previously described [9], FITC-labeled albumin (MW = 69-70 kDa, Sigma, St. Louis, MO, USA) assay was performed for visualization of

BBB leakage. Three days after MPTP or saline injections, mice were intranscardially perfused with Hank's Balanced Salt Solution containing heparin (10 U/mL) and then immediately by 5 mL FITC-labeled albumin (5 mg/mL, in 0.1 M phosphate-buffered saline (PBS) buffer) injected at a rate of 1.5 mL/minute. Brains were dissected from the skull, postfixed overnight in buffered 4% paraformaldehyde at 4°C. After fixation, the brains were cut into 30 μm slices using a sliding microtome. Sections were mounted on gelatin-coated slides, and the FITC-labeled albumin contained vessels that were examined by confocal microscopy (Carl Zeiss). To determine the total area for FITC-labeled albumin leakage, 3 or 4 images of SN region were obtained, thresholded using Imag J, quantified, and normalized by value of PBS injected MMP-$3^{+/+}$ mice.

2.7. Statistical Analysis. All values are expressed as mean ± SEM. Statistical significance ($P < 0.05$ for all analyses) was assessed by two-way ANOVA using Instat 3.05 (GraphPad Software, San Diego, CA, USA) followed by Bonferroni post hoc test.

3. Results

3.1. MMP-3 Deficiency Protects Nigrostriatal DA Neurons from MPTP Neurotoxicity. Mice in each group (MMP-$3^{+/+}$ or MMP-$3^{-/-}$ mice) received four intraperitoneal injections of MPTP (20 mg/kg) or PBS (control) at 2-hour intervals. Seven days later, brains were removed, and sections were immunostained for TH to specifically detect DA neurons. Consistent with our recent reports [24–26], there was a significant loss of TH-immunopositive (ip) cell bodies in the SNpc (Figure 1(b)) and of TH-ip fibers in the STR (Figure 2(b)) at 7 days in MPTP-injected MMP-$3^{+/+}$ mice compared with PBS-treated MMP-$3^{+/+}$ control mice (Figures 1(a) and 2(a)). TH-ip cells in the SNpc and TH-ip fibers in the STR were quantified by stereological counts and densitometric analyses, respectively. The results showed that MPTP treatment reduced the number of TH-ip neurons by 59% (Figure 1(e), $P < 0.001$) and decreased the optical density (OD) of TH-ip fibers by 64% (Figure 2(e), $P < 0.001$) compared with PBS-treated MMP-$3^{+/+}$ control mice.

In contrast to the above, the results of TH immunohistochemistry demonstrated that in MMP-3-deficient (MMP-$3^{-/-}$) mice, the loss of TH-ip cell bodies in the SNpc (Figure 1(d)) and TH-ip fibers in the STR (Figure 2(d)) was significantly reduced compared to that seen in MPTP-treated MMP-$3^{+/+}$ mice (Figures 1(b) and 2(b)). Stereological counts and densitometric analyses showed that the number of TH-ip neurons in the SNpc and the OD in the STR was higher by 30% (Figure 1(e); $P < 0.05$) and 31% (Figure 2(e); $P < 0.05$), respectively, in MMP-$3^{-/-}$ mice compared to MMP-$3^{+/+}$ mice. These data confirm our previous finding showing that MMP-3 participates in MPTP-induced neurotoxicity, resulting in the degeneration of nigrostriatal DA neurons *in vivo* [23].

3.2. MMP-3 Deficiency Improves Motor Behavior in MPTP Mice. We next used two different paradigms of rotarod performance to examine if MMP-3 affects MPTP-induced motor behavior [25, 29]. Animals receiving the different treatment regimens were evaluated 7 days after the last MPTP injection by measuring the fall time latency for the fixed mode (20 rpm for 20 min) and for the accelerating mode (4–40 rpm for 5 min) one day after carrying out the fixed mode evaluation. In MMP-$3^{+/+}$ mice, the MPTP treatment decreased the sustained rotarod time in the fixed mode to 10.41 ± 0.78 min (representing a 48% decrease; $P < 0.001$; Figure 3(a)) and to 2.93 ± 0.91 min (representing a 37% decrease) in the accelerating mode ($P < 0.001$; Figure 3(b)), compared with control (PBS treatment). In contrast, this behavioral dysfunction in MPTP-treated MMP-$3^{+/+}$ mice was partially reduced in MMP-$3^{-/-}$ mice, which exhibited increased falling latencies of 18.52 ± 0.91 min ($P < 0.05$; Figure 3(a)) in the fixed mode and 4.03 ± 0.41 min ($P < 0.05$; Figure 3(b)) in the accelerating mode, respectively. The falling latency in both paradigms was not significantly different between PBS-injected MMP-$3^{-/-}$ mice and their wild-type littermates.

3.3. MMP-3 Deficiency Prevents MPTP-Induced BBB Damage In Vivo. Increased BBB permeability has been observed in the midbrains of PD patients [6, 30]. In this regard, several *in vivo* studies have also demonstrated that increased BBB permeability can play an important role in the induction of DA neuronal death in the MPTP mouse model of PD [8, 31]. Thus, we next investigated whether MMP-3 affects MPTP-induced BBB disruption by detecting FITC-labeled albumin in the brain three days after the last MPTP treatment. In PBS-injected MMP-$3^{+/+}$ (Figure 4(a)) and MMP-$3^{-/-}$ mice (Figure 4(c)), FITC-labeled albumin was confined to the blood vessels of the SN *in vivo*, indicating that the BBB was intact. However, in the SN of MPTP-treated MMP-$3^{+/+}$ mice, the diffusion of FITC-labeled albumin into the brain from multiple blood vessels was clearly evident ($P < 0.01$; Figures 4(b) and 4(e)). In contrast, the vascular diffusion of FITC-labeled albumin was not as clear-cut in MPTP-treated MMP-$3^{-/-}$ mice ($P < 0.05$; Figures 4(d) and 4(e)). These results suggest that MMP-3 could be involved in an MPTP-induced damage to the BBB.

3.4. MMP-3 Deficiency Inhibits Microglial Activation and the Infiltration of T Cells into the SN In Vivo. Accumulating evidence, including that from our group, suggests that activated microglia play an important role in DA neuronal cell death in the MPTP mouse model [3, 32]. Accordingly, we next examined if the resistance to MPTP neurotoxicity exhibited by MMP-3-deficient mice could result from the inhibition of microglial activation in the SN. Three days after the last MPTP injection, sections of brain tissue from mice were immunostained with ED-1 antibody, a marker for activated microglia. In contrast to the SN of control mice (MMP-$3^{+/+}$ mice) treated with PBS (Figure 5(a)), where relatively few ED-1-ip microglia were observed, the SN of MPTP-treated MMP-$3^{+/+}$ mice contained numerous ED-1-ip cells. The majority

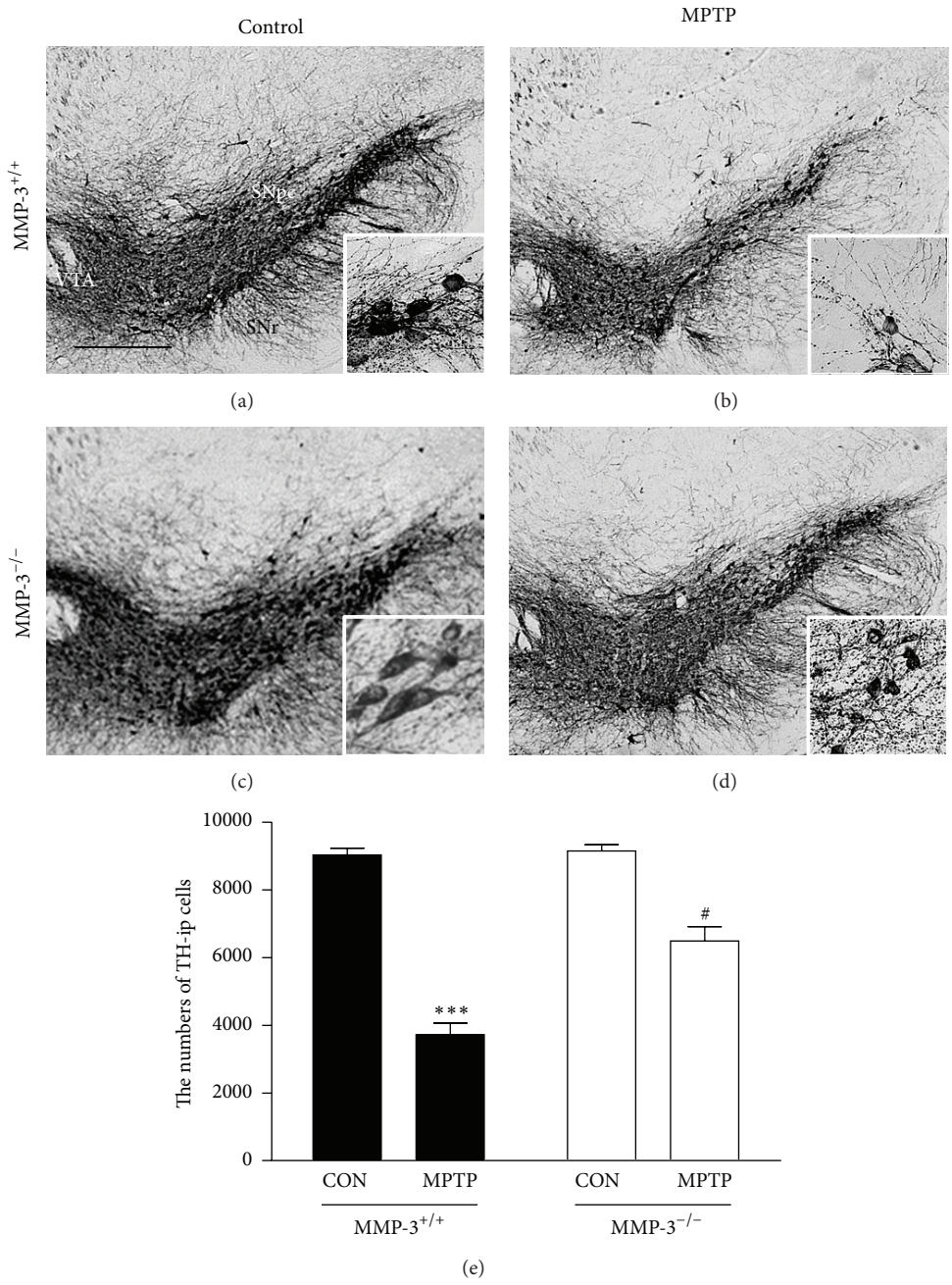

Figure 1: MPTP-induced neurotoxicity is attenuated in the SNpc of MMP-3$^{-/-}$ mouse brain. (a) Animals (MMP-3$^{+/+}$ or MMP-3$^{-/-}$ mice) receiving PBS as a control (a and c) and MPTP (b and d) were sacrificed 7 days after the last MPTP injection. *Insets*, higher magnifications of (a–c). The brain tissues were cut into 30 μm thick coronal sections using a sliding microtome and immunostained with an antibody against the DA neuronal marker TH. Scale bar, 300 μm. (e) Bars represent the number of TH-ip neurons in the SN after indicated treatment in the absence (MMP-3$^{-/-}$) or presence (MMP-3$^{+/+}$) of MMP-3. Five to six animals were used for each experimental group. Two-way ANOVA with Bonferroni post hoc test ($F(1, 15) = 17.57$, $P < 0.001$), $^{***}P < 0.001$, significantly different from PBS-injected MMP-3$^{+/+}$ mice; $^{\#}P < 0.05$, significantly different from MPTP-injected MMP-3$^{+/+}$ mice. SNpc, substantia nigra pars compacta; VTA, ventral tegmental area; SNr, substantia nigra reticulata.

of ED-1-ip microglia in the MPTP-treated SN of MMP-3$^{+/+}$ mice displayed an activated morphology, including larger cell bodies with short and thick processes or no processes at all (Figure 5(b)). In contrast, the number of ED-1-ip-activated microglia was clearly decreased in the SN of MPTP-treated MMP-3$^{-/-}$ mice (Figures 5(c) and 5(g)).

Recent studies have shown that the migration of peripheral T lymphocytes within the CNS is associated with DA neuronal death in the SN of PD patients and of MPTP-treated mice [5, 33]. Accordingly, we examined whether a deficiency of MMP-3 inhibits the infiltration of T lymphocytes. As described previously [32], the MPTP-induced infiltration of

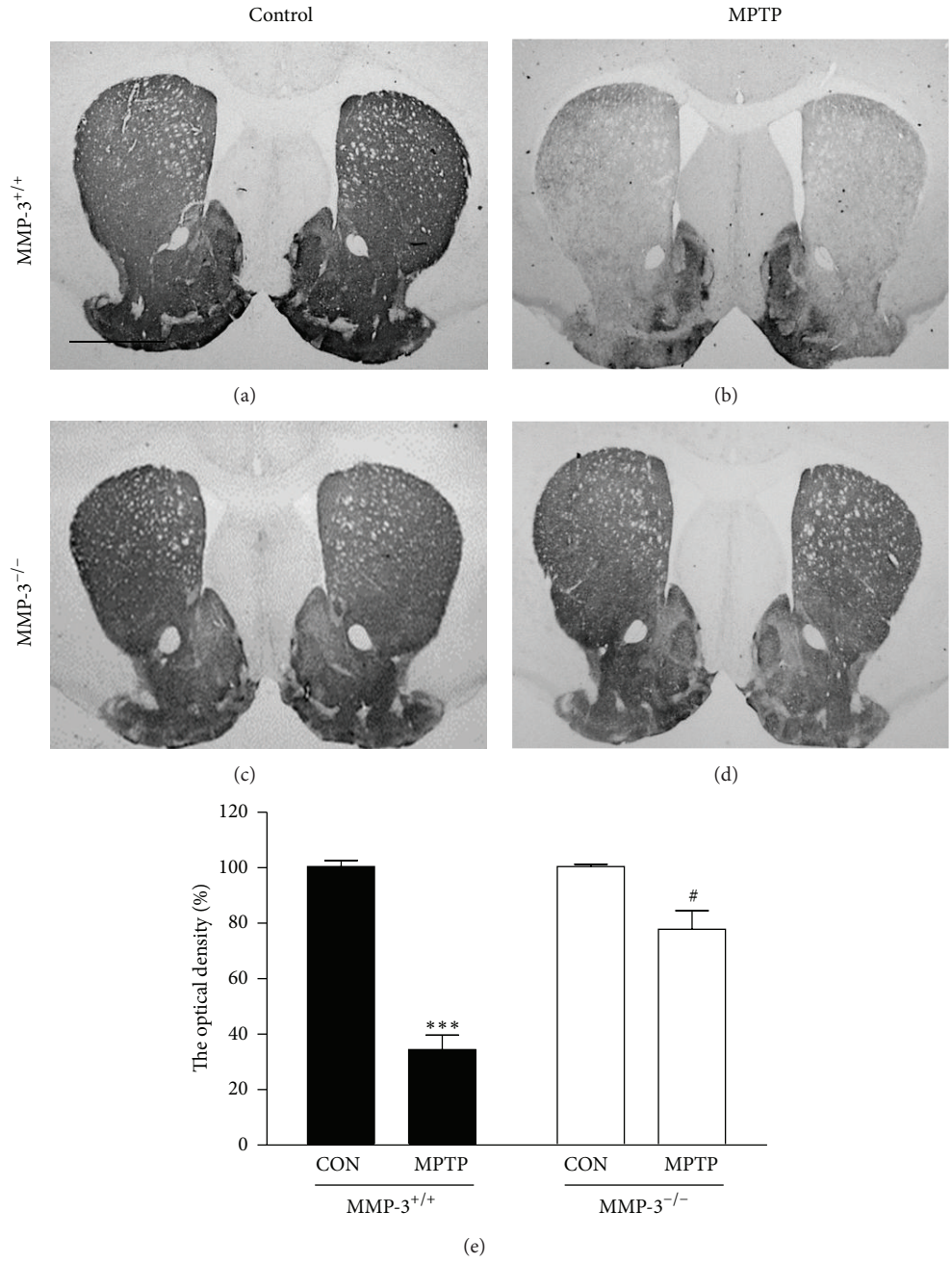

Figure 2: MPTP-induced neurotoxicity is attenuated in the striatum of MMP-$3^{-/-}$ mouse brain. Striatal tissues obtained from the same animals as used in Figure 1 were immunostained with TH antibody for DA fibers. Animals were treated with PBS as a control (a and c) and MPTP (b and d). (e) Bars represent optical density of TH-ip fibers in the striatum. Scale bar, 250 μm. Five to six animals were used for each experimental group. Two-way ANOVA with Bonferroni post hoc test ($F(1, 15) = 22.22$, $P < 0.001$), $^{***}P < 0.001$, significantly different from PBS-injected MMP-$3^{+/+}$ mice; $^{\#}P < 0.05$, significantly different from MPTP-injected MMP-$3^{+/+}$ mice.

T cells was visualized by CD-3 immunostaining of sections adjacent to those used for ED-1 immunostaining. Compared with the SN of PBS-treated MMP-$3^{+/+}$ mice (Figure 5(d)), a 14-fold increase in the number of CD-3-ip cells in the SN of MPTP-treated MMP-$3^{+/+}$ mice was seen three days after the last MPTP injection ($P < 0.001$; Figures 5(e) and 5(g)). By comparison, the number of CD-3-ip cells in the SN of MMP-$3^{-/-}$ mice was 79% lower than that in the MMP-$3^{+/+}$ mice ($P < 0.05$; Figures 5(f) and 5(g)). Stereological cell counting showed that the numbers of ED-1 and CD-3-ip cells in the SN were similar between PBS-injected MMP-$3^{-/-}$ mice and their wild-type littermates (Figure 5(g)).

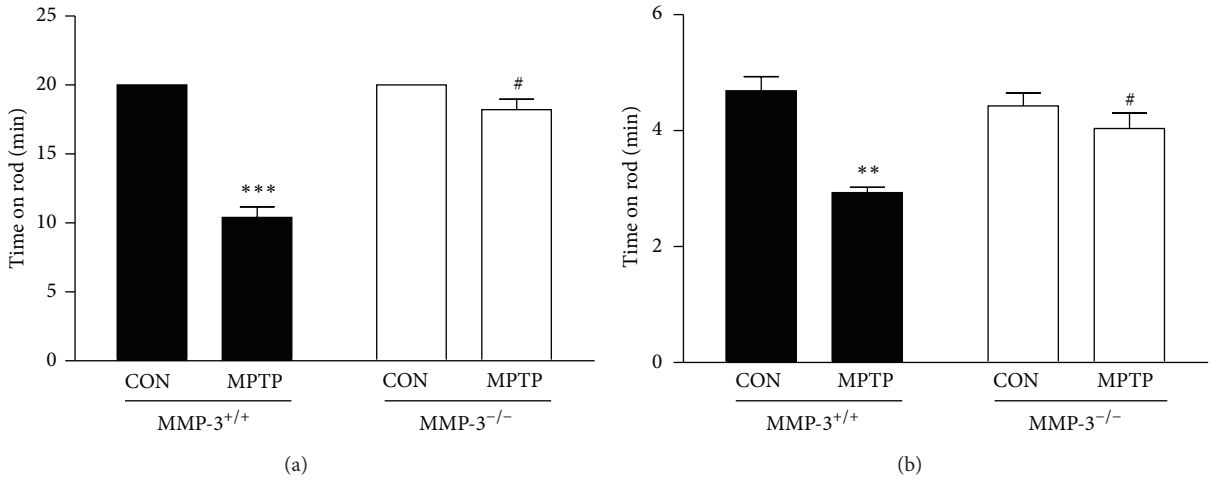

Figure 3: Effects of MMP-3 on motor performance in MPTP-treated mice. (a) After 7 days from MPTP injection, mice were subjected to rotating rod (20 rpm) for 20 min, and falling time was recorded. (b) Next day, mice were placed on an accelerating rotating rod, and the maximum time before the mouse fell from the rod was recorded. Five to six animals were used for each experimental group. Two way ANOVA with Bonferroni post hoc test, fixed mode; ($F(1, 15) = 45.67$, $P < 0.001$), accelerating mode; ($F(1, 15) = 9.73$, $P < 0.01$), $^{***}P < 0.001$ and $^{**}P < 0.01$, significantly different from PBS-injected MMP-$3^{+/+}$ mice; $^{\#}P < 0.05$, significantly different from MPTP injected MMP-$3^{+/+}$ mice.

4. Discussion

Accumulating evidence suggests that MMP-3 is associated with DA neuronal death and neuroinflammation and as a consequence is involved in the pathogenesis of PD [10, 34]. Several studies have previously shown that MMP-3 plays an important role in caspase signaling under ER stress [35] or BH-4- [36] induced apoptotic DA neuronal death and is involved in the induction of reactive oxygen species (ROS) and proinflammatory cytokine production in activated microglia [15]. The pharmacological inhibition of MMP-3 recovers motor deficits and suppresses microglia activation in MPTP-treated mice [37]. We have shown here, for the first time, that in addition to degeneration of DA neurons and microglial activation, MMP-3 participates in the impairment of BBB integrity and T-leukocyte infiltration into the SN of MPTP-treated MMP-$3^{+/+}$ mice.

Neuroinflammation can be produced by many factors, including activated microglia, BBB disruption, and infiltration of peripheral immune cells into the brain and as such plays a critical role in the CNS immune system [38]. Intrinsic immune mechanisms perform neuroprotective and supportive functions in the normal CNS; however, under neuropathological conditions, neuroinflammation may be triggered by transient initiation factors such as neuronal damage, thereby contributing to irreversible DA neuronal death in the SN and locomotor deficits [39].

The pathogenesis of PD has been linked increasingly to neuroinflammation and BBB impairment [40–42]. Neuroinflammation was found to exert harmful effects on BBB integrity [43], and BBB leakage has been demonstrated in PD patients [6, 30] and in MPTP- or 6-OHDA-treated animal models of PD [9, 44]. Because the BBB helps regulate and protect the microenvironment of the brain and its disruption results in the loss of DA neurons [45], it has been hypothesized that BBB dysfunction may account for, at least in part the degeneration of DA neurons in PD [46, 47].

The BBB, which is comprised of neurovascular units such as endothelial cells, pericytes, neurons, and astrocyte endfeet, restricts the entry of plasma components, blood cells, and leukocytes into the brain. When these infiltrate into the brain parenchyma due to neurodegenerative processes or neuroinflammation, neurotoxic substances can be produced that cause neuronal dysfunction and loss [43]. We recently demonstrated in LPS-treated rats and MPTP-treated mice that compromising the integrity of the BBB contributes to the degeneration of nigrostriatal DA neurons in the SN [32]. The present data show that MPTP increases the infiltration of FITC-labeled albumin from blood vessels into the SN and that genetic deletion of the MMP-3 (i.e., MMP-$3^{-/-}$ mice) likely attenuates the MPTP-induced damage to the BBB and subsequent impact on the SN.

Glial cell activation is one of the major contributors to neuroinflammation and is implicated in the pathogenesis and progression of PD [32, 38]. In particular, activated microglia can release harmful substances such as proinflammatory cytokines, ROS, and reactive nitrogen species which subsequently cause neuronal loss and dysfunction in a range of neurodegenerative diseases [3]. Several studies have demonstrated that reactive microglia expressing proinflammatory mediators are present in the midbrains of PD patients [48–50], while increasing evidence, including our own [24–26], has shown that activated microglia contribute to DA neuronal cell death through NADPH- [50] and MPO- [48] mediated oxidative stress and production of proinflammatory molecules (iNOS, TNF-α, and IL-1β) [51] in the MPTP mouse model of PD. Recently, we reported that the number of CD-11b- and Iba-1-ip-activated microglia was correlated with ED-1-ip microglia/macrophage phagocytotic activity in the SN

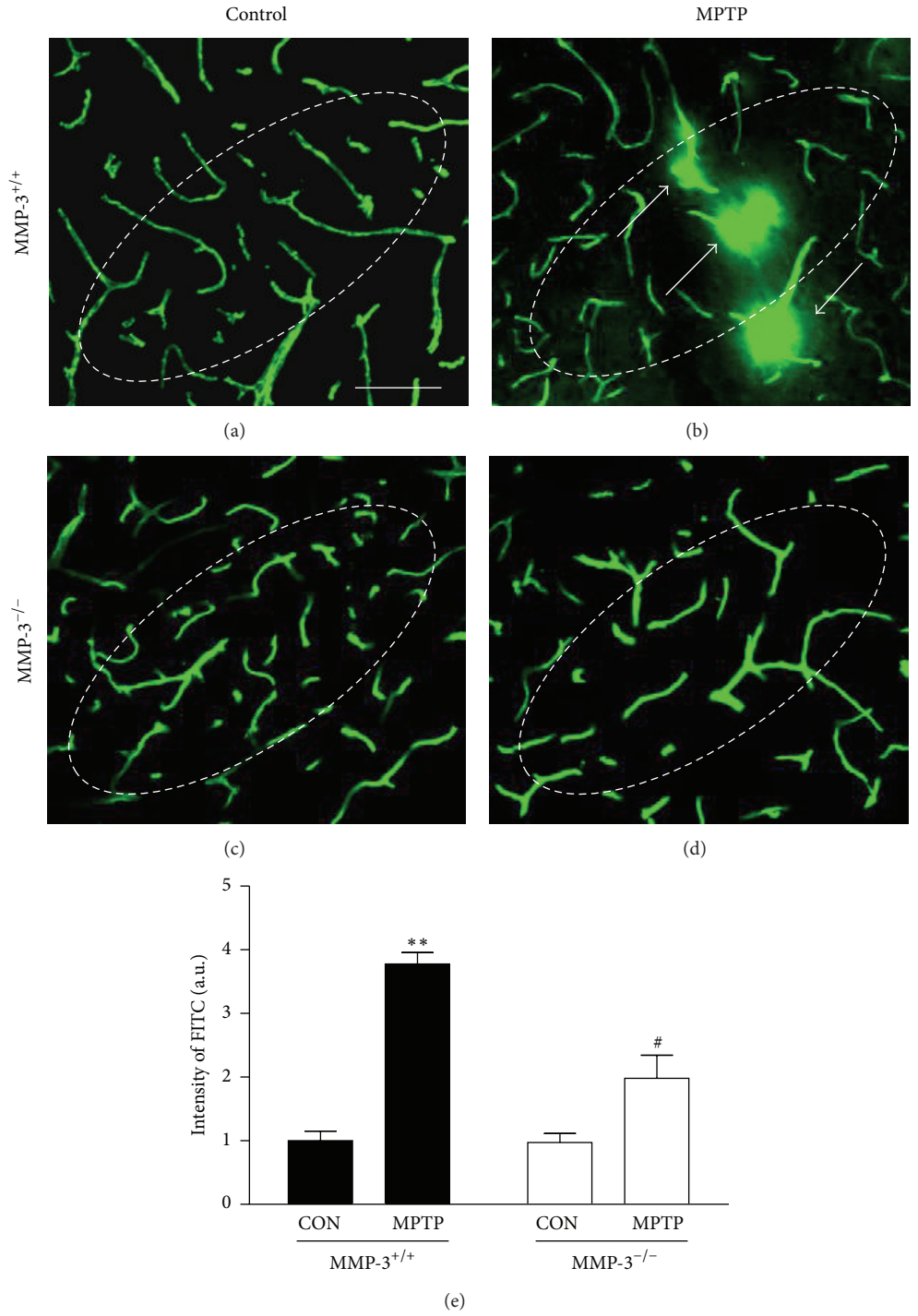

FIGURE 4: MPTP-induced BBB breakage is prevented in MMP-$3^{-/-}$ mice. At 3 days after MPTP injection in the absence (MMP-$3^{-/-}$; (c and d)) or presence (MMP-$3^{+/+}$; (a and b)) of MMP-3, FITC-linked albumin was administered to detect for brain vascular permeability. (e) Bars represent the FITC-labeled albumin-positive area in the SNpc, respectively. Four or five animals were used for each experimental group. Whole values are normalized by PBS-injected MMP-$3^{+/+}$ mice. Two-way ANOVA with Bonferroni post hoc test ($F(1, 15) = 10.19$, $P < 0.001$). $^{**}P < 0.01$, significantly different from PBS-injected MMP-$3^{+/+}$ mice; $^{\#}P < 0.05$, significantly different from MPTP-injected MMP-$3^{+/+}$ mice. Arrows indicate FITC-linked albumin leakage, indicating extravasations of FITC-labeled albumin into the brain. Dotted lines indicate the SNpc, where DA neurons were degenerating after MPTP injection. Scale bar, 400 μm.

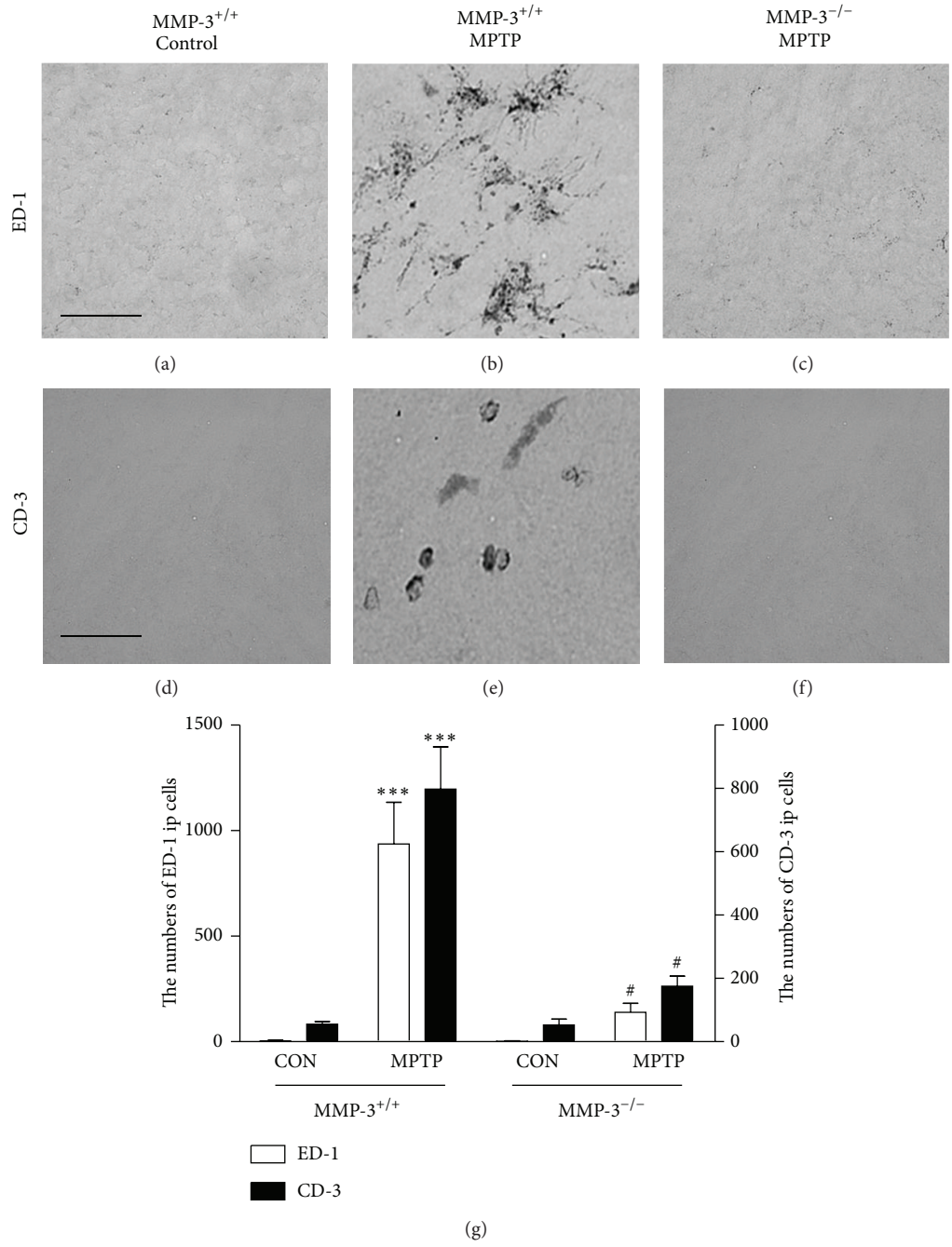

Figure 5: MPTP-induced increases in ED-1 and CD-3-ip cells are suppressed in the SNpc of MMP-3$^{-/-}$ mouse brain. ((a–f)) Sections adjacent to those used for FITC-linked albumin staining were examined for ED-1 or CD-3 immunostaining. (g) Bars represent the numbers of ED-1 or CD-3-ip cells in the SNpc, respectively. Four or five animals were used for each experimental group. Dotted lines indicate the SNpc. Scale bar, 100 μm. Two-way ANOVA with Bonferroni post hoc test, ED-1-ip cells; ($F(1, 15) = 45.67$, $P < 0.001$), CD-3-ip cells; ($F(1, 15) = 13.72$, $P < 0.01$), $^{***}P < 0.001$, significantly different from PBS-injected MMP-3$^{+/+}$ mice; $^{\#}P < 0.05$, significantly different from MPTP-injected MMP-3$^{+/+}$ mice.

of MPTP-treated mice [26]. With respect to that finding, the present study shows that MPTP significantly upregulated the expression of ED-1-ip microglia in the SN. In contrast, the increase in number of ED-1-ip cells was dramatically attenuated in the MPTP-treated SN of MMP-3 knockout (MMP-3$^{-/-}$) mice. These data support the hypothesis that MMP-3 has the capacity to induce microglial activation, resulting in the degeneration of DA neurons.

In addition to microglial activation, the infiltration of T leukocytes may be involved in nigrostriatal DA neuronal death. Several lines of evidence highlight the presence of infiltrating T cells (CD-4- or CD-8-ip cells) in the SN of PD

patients [5] and MPTP-treated mice [5, 33]. Brochard and colleagues also showed the CD-4- but not CD-8-mediated degeneration of DA neurons in the SN of MPTP-treated mice [5]. Depboylu and colleagues recently showed that infiltrating CD-3-ip T lymphocytes, representing both CD-4-ip and CD-8-ip cells, are implicated in the regulation of the adaptive immune system through crosstalk with microglia and/or macrophages in the SN in the MPTP mouse model of PD [33]. Moreover, CD-4-ip T cells mediated (via the Fas/Fas ligand pathway) the detrimental effects on DA neurons in the SN of MPTP-treated mice [5]. In this way, the data in this report show that MPTP increases the number of infiltrating CD-3-ip T cells in the SN of MMP-$3^{+/+}$ control mice; this number was significantly reduced in the SN of MPTP-treated MMP-$3^{-/-}$ mice, eventually leading to the improved survival of DA neurons. These data suggest that MMP-3-induced T-cell infiltration contributes to MMP-3's neurotoxic effect on DA neurons in the SN.

The predominant behavioral change in the MPTP-treated mice was the reduced latency to fall from the rotarod apparatus, thereby reflecting diminished coordination and balance [24–26]. The two different designs of rotarod performance (fixed mode and accelerating mode) indicate that nigrostriatal DA neuron loss is well correlated with motor dysfunction on this apparatus. Consistent with our recent data [24, 26], MPTP reduced the latency to fall from the rotarod apparatus in MMP-$3^{+/+}$ control mice. This behavioral dysfunction was partially improved in MPTP-treated MMP-$3^{-/-}$ mice. These behavioral effects of MMP-3 deletion on the lesioned nigrostriatal DA system, together with the knowledge that the genetic ablation of MMP-3 prevents microglial activation, infiltration of T leukocyte, and BBB disruption, suggest that MMP-3 could be a useful therapeutic target for treating PD and other neurodegenerative disorders involving neuroinflammation and compromised BBB integrity.

5. Conclusion

The present study shows that matrix metalloproteinase-3 (MMP-3) participates in degeneration of nigrostriatal dopaminergic neurons in the MPTP model of Parkinson's disease by neuroinflammation-mediated BBB disruption and infiltration of T leukocytes. To our knowledge, this is the first study to demonstrate that neurotoxic effects of MMP-3 in the MPTP-treated SN are associated with the ability of MMP-3 to increase BBB damages, microglia/macrophage phagocytosis, and infiltration of T leukocytes, suggesting that MMP-3 can be novel therapeutic target for PD and other disorders associated with BBB integrity and neuroinflammation.

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Research Article

Activation of Protease-Activated Receptor 2-Mediated Signaling by Mast Cell Tryptase Modulates Cytokine Production in Primary Cultured Astrocytes

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Protease-activated receptor 2 (PAR-2), which is abundantly expressed in astrocytes, is known to play major roles in brain inflammation. However, the influence of the natural agonist of PAR-2, tryptase, on proinflammatory mediator released from astrocytes remains uninvestigated. In the present study, we found that tryptase at lower concentrations modestly reduced intracellular ROS production but significantly increased IL-6 and TNF-α secretion at higher concentrations without affecting astrocytic viability and proliferation. The actions of tryptase were alleviated by specific PAR-2 antagonist FSLLRY-NH2 (FS), indicating that the actions of tryptase were via PAR-2. PI3K/AKT inhibitor LY294002 reversed the effect of tryptase on IL-6 production, whereas inhibitors specific for p38, JNK, and ERK1/2 abolished the effect of tryptase on TNF-α production, suggesting that different signaling pathways are involved. Moreover, tryptase-induced activation of MAPKs and AKT was eliminated by FS, implicating that PAR-2 is responsible for transmitting tryptase biosignals to MAPKs and AKT. Tryptase provoked also expression of TGF-β and CNTF in astrocytes. The present findings suggest for the first time that tryptase can regulate the release of cytokines from astrocytes via PAR-2-MAPKs or PAR-2-PI3K/AKT signaling pathways, which reveals PAR-2 as a new target actively participating in the regulation of astrocytic functions.

1. Introduction

As a unique family of G protein-coupled receptors, newfound protease-activated receptors (PARs) are widely expressed on the cells in central nervous system (CNS), including neurons and glial cells [1], regulating cell responses to extracellular serine proteases as cell surface sensors and contributing extensively to the regulation of homeostasis as well as to the dysfunctional responses of these cells required for progression of cerebral diseases [2]. Among the four PARs identified to date, PAR-2 is a unique one activated by trypsin and mast cell tryptase while others (PAR-1, -3, and -4) activated by thrombin [3]. The role of PAR-2, which is distributed extensively throughout the nervous system (including CNS and peripheral nervous system), has been principally investigated in peripheral nervous system, where it is known to play major roles in injury, inflammation, neuronal signaling, and nociception [4, 5]. And the physiological role of PAR-2 in CNS remains unclear but its activation has been shown to increase intracellular Ca^{2+} levels in both neurons and astrocytes [6, 7] as well as trigger the release of gliotransmitters such as GRO/CINC-1 [8–10] and nitric oxide [11]. Recent group of evidence have revealed that PAR-2 contributes to neuroprotection and/or neurodegeneration in the brain under pathological conditions [12–15]. Therefore, PAR-2 has been suggested to be a novel therapeutic target for the treatment of brain disorders.

Tryptase, the major secretory protein of mast cells, is the natural agonist of PAR-2 and can stimulate peripheral mononuclear cells to secrete tumor necrosis factor-alpha (TNF-α)

and interleukin-6 (IL-6) [16] to induce widespread inflammation [17]. Although mast cells typically reside at barrier sites of the body such as the intestinal mucosa and blood brain barrier (BBB) [18], Silverman et al. found that mast cells can rapidly penetrate brain blood vessels and migrate into the neural parenchyma [19], implying an interaction between mast cells and nerve tissue cells. As the most abundant cells in brain parenchyma, astrocytes play pivotal roles in BBB integrity and CNS function such as synapse formation [20], communication [21], cerebrovascular tone [22], adult neurogenesis [23], as well as neuroimmune [24]. Since PAR-2 is widely expressed in astrocytes and is recognized for the modulatory properties of neuroinflammation and neurodegeneration such as multiple sclerosis [25], its contribution to astrocytic functions remains to be elucidated. In the present study, we investigated the consequence of tryptase stimulation on (1) the astrocytic survival and proliferation; (2) the production of IL-6, TNF-α, and reactive oxygen species (ROS); (3) the involvement of MAPKs and PI3K/AKT pathways in PAR-2 activation; (4) the levels of potent neural cytokines transforming growth factor-β (TGF-β) and ciliary neurotrophic factor (CNTF) generated by astrocytes.

2. Materials and Methods

2.1. Reagents. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco-BRL (Grand Island, NY, USA). Poly-D-lysine, tryptase, SB203580, PD98059, SP600125, and LY294002 were purchased from Sigma-Aldrich (St. Louis, MO, USA). PAR-2 inhibitor FS were synthesized by CL Bio-Scientific Inc. (Xi An, China). Dojindo Cell Counting Kit-8 was purchased from Sigma-Aldrich (St. Louis, MO, USA). Rat IL-6 Immunoassay Kit and Rat TNF-α Immunoassay Kit were obtained from R&D Systems, Inc. (Minneapolis, MN, USA). LIVE green reactive oxygen species detection kit was purchased from Molecular Probes Invitrogen (Carlsbad, CA, USA). Specific glial fibrillary acid protein (GFAP) antibody (a marker for astrocytes) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Specific monoclonal antibodies against p38, phospho-p38, SAPK/JNK (c-JUN N-terminal kinase), phospho-SAPK/JNK, p44/42 MAPK (extracellular regulated protein kinases, ERK), phospho-p44/42 MAPK (phospho-ERK) and AKT, and phospho-AKT were obtained from Cell Signaling (Beverly, MA, USA). Specific polyclonal antibodies against TGF-β and CNTF were purchased from Abcam (Cambridge, MA, UK).

2.2. Primary Astrocyte Cultures. Confluent primary astrocyte cultures were prepared from Sprague-Dawley rats as previously described with slight modification. All animal procedures were performed according to the NIH Guide for Animal Care and approved by the institutional animal care and use committee. Briefly, postnatal (P1-P2) rats were killed by rapid decapitation, cerebral cortices were triturated and cells were plated on poly-D-lysine precoated culture flasks in DMEM, containing 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2/95% air. Culture medium was replaced 24 h later and then changed every 2-3 days. After reaching a confluent monolayer of cells (10–14 days), microglia were eliminated from astrocytes by shaking off for 5 h at 100 r.p.m. and astrocytes were replated in poly-D-lysine coated culture dishes, 96-well or 6-well plates. The enriched astrocytes were >98% pure as determined by astrocytic marker GFAP.

2.3. Cell Proliferation Assay. Cell viability was measured by conversion of Dojindo's highly water-soluble tetrazolium salt WST-8 to a yellow-colored water-soluble formazan (CCK8 assay). The amount of formazan dye generated by the activity of mitochondrial dehydrogenases in cells is directly proportional to the number of living cells. CCK8 is more sensitive than the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay [26]. Cells were collected and seeded in 96-well plates at a density of 10^5 cells/cm^2. After incubation for 48 h, cells were exposed to fresh medium containing various concentrations of tryptase (0.001, 0.01, 0.1, 1, and 10 μg/mL) at 37°C for further 24 h. Then, 20 μL of CCK8 solution in PBS was added to each well and the plates were incubated for an additional 2 h. The optical density of each well was measured using a microculture plate reader at a 450 nm wavelength.

2.4. Intracellular Reactive Oxygen Species Assay. The production of intracellular ROS was analyzed by 2′,7′-dichlorodihydrofluorescein (DCFH) oxidation. The 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) reagent passively enters cell where it is deacetylated by esterase to nonfluorescent DCFH. Inside the cell, DCFH reacts with ROS to form 2′,7′-dichlorofluorescein (DCF), the fluorescent product. For this assay, 10 mM of DCFH-DA was dissolved in DMSO and was diluted 500-fold in HBSS to give 20 μM of DCFH-DA. Enriched-astrocyte cultures seeded (5×10^4) in 96-well plates were then exposed to DCFH-DA for 1 h at 37°C in dark, followed by treatment with HBSS containing various concentrations of tryptase for 2 h. After being rinsed twice with PBS, green fluorescence from DCF in cells was measured in the FL1 Log channel through a 525-nm band-pass filter on the Coulter EPICS XL/X1-MCL (Beckman Coulter Company, Miami, FL, USA).

2.5. IL-6 and TNF-α Assay. The amount of IL-6 and TNF-α in the culture medium was measured with commercial ELISA kits from R&D Systems, respectively.

2.6. Western Blot Analysis. Cells were collected and homogenized in 200 μL lysing buffer. After incubation for 20 min on ice, cell lysate was centrifuged at 12,000 g at 4°C for 10 min and protein concentration in the extracts was determined by the Bradford assay. Proteins in cell extracts were denatured with sodium dodecyl sulfate (SDS) sample buffer and separated by 10% SDS-polyacrylamide gel at 80 V for 2 h. Then proteins were electrotransferred to nitrocellulose membranes at 300 mA for various time points by using a Bio-Rad miniprotein-III wet transfer unit. The membranes were blocked with 5% BSA dissolved in Tris-buffered saline with

Tween 20 (TBST) (pH 7.5, 10 mM Tris-HCl, 150 mM NaCl and 0.1% Tween 20) at room temperature for 1 h. This was followed by incubating the membranes with different antibodies (anti-p38, -phospho-p38; -JNK, -phospho-JNK; -ERK, -phospho-ERK; -AKT, -phospho-AKT; -TGF-β at 1 : 800 dilution and -CNTF at 1 : 1500 dilution) overnight at 4°C, and finally incubated with a horseradish peroxidase-conjugated anti-rabbit IgG for 1 h at room temperature. Protein bands on the membranes were visualized by an enhanced chemiluminescence kit.

2.7. Statistical Analysis. All values shown are presented as means ± SEM. The significance of the difference between control and samples treated with various drugs was determined by one-way ANOVA followed by the post-hoc least significant difference test. Differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. Tryptase Had No Effect on the Astrocytic Viability. Cell survival measured by CCK8 analysis revealed that incubation with different dose of tryptase (0.001, 0.01, 0.1, 1, and 10 μg/mL) for 24 h had no significant effect on astrocytic viability and proliferation. No impact was either observed in specific PAR-2 inhibitor FS (200 or 400 μM), p38 inhibitor SB203580 (20 μM), JNK inhibitor SP600125 (20 μM), ERK1/2 inhibitor PD98059 (20 μM) and PI3K/AKT inhibitor LY294002 (20 μM) (Figure 1).

3.2. Tryptase Inhibits ROS Production in Primary Cultured Astrocytes via PAR-2. The results of the DCF assay indicated that incubation with tryptase at low concentrations (0.001 and 0.01 μg/mL) for 2 h modestly inhibited the intracellular levels of ROS, which was abolished by FS (400 μM) (Figure 2). The fluorescence of DCF in cells decreased to 83% (0.001 μg/mL group) and 86% (0.01 μg/mL group) of that in the control, and specific PAR-2 inhibitor FS diminished the effects of tryptase (0.001 μg/mL) on ROS generation in astrocytes, implying that activation of PAR-2 at low concentrations of tryptase is responsible for the inhibition of ROS production.

3.3. Tryptase Regulated IL-6 and TNF-α Secretion from Primary Cultured Astrocytes via PAR-2. As shown in Figure 3, incubation with tryptase at the dose of 0.001, 0.01, 0.1, 1, and 10 μg/mL for 24 h produced a concentration dependent increase in IL-6 secretion from primary cultured astrocytes with a minimum effective dose of 0.1 μg/mL (Figure 3(a)). Meanwhile, tryptase (1 and 10 μg/mL) also increased TNF-α secretion at a minimum effective dose of 1 μg/mL (Figure 3(b)), but modestly inhibited TNF-α secretion at the dose of 0.001 μg/mL. PAR-2 inhibitor FS (200 and 400 μM) was able to diminish tryptase (1 μg/mL) induced IL-6 and TNF-α increase but itself alone (200 and 400 μM) failed to affect IL-6 and TNF-α secretion from astrocytes (Figures 3(a) and 3(b)), suggesting that tryptase is able to modulate the secretion of IL-6 and TNF-α from astrocytes via PAR-2.

3.4. Tryptase Regulated TNF-α but Not IL-6 Secretion via MAPKs from Primary Cultured Astrocytes. To investigate the involvement of MAPKs and PI3K/AKT signaling pathways in the IL-6 and TNF-α secretion, we used pharmacological inhibitors of MAPKs and PI3K/AKT. SB203580 is a pyridinyl imidazole compound which acts as a competitive inhibitor of ATP binding on the p38 kinase and thus serves as a specific inhibitor of p38 MAPKs. PD98059 is a potent, selective, and cell-permeable inhibitor of MEK1, which results in inhibition of the phosphorylation and activation of ERK1/2. SP600126 is a potent, selective, reversible, and cell-permeable inhibitor of JNK, a Ser/Thr kinase that phosphorylates c-jun. LY294002 is a potent, selective, cell permeable, and specific inhibitor of PI3K/AKT. As shown in Figure 4, SB203580

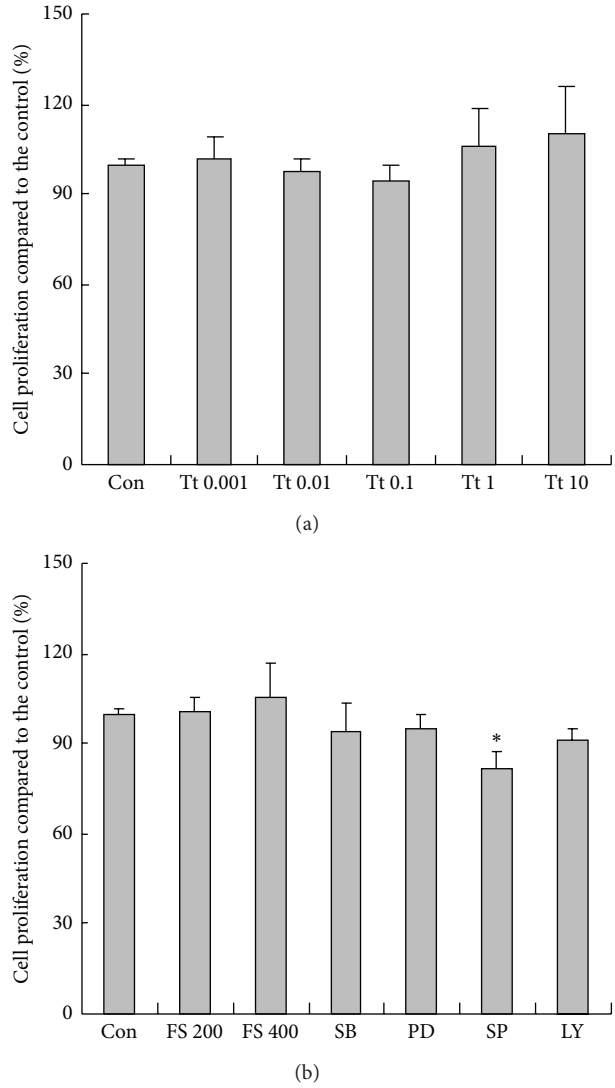

Figure 1: Effects of tryptase (Tt) and specific inhibitor of PAR-2 or MAPKs on the viability of astrocytes. For the dose-dependent studies astrocytes were treated with either culture medium only or various concentrations of tryptase (0.001, 0.01, 0.1, 1, and 10 μg/mL), FSLLRY-NH2 (FS, 200 or 400 μM), SB203580 (SB, 20 μM), PD98059 (PD, 20 μM), SP600125 (SP, 20 μM) and LY294002 (LY, 20 μM) for 24 h. Data are presented as the mean ± SEM in triplicate on four separate occasions. $^*P < 0.05$ versus control groups (Con).

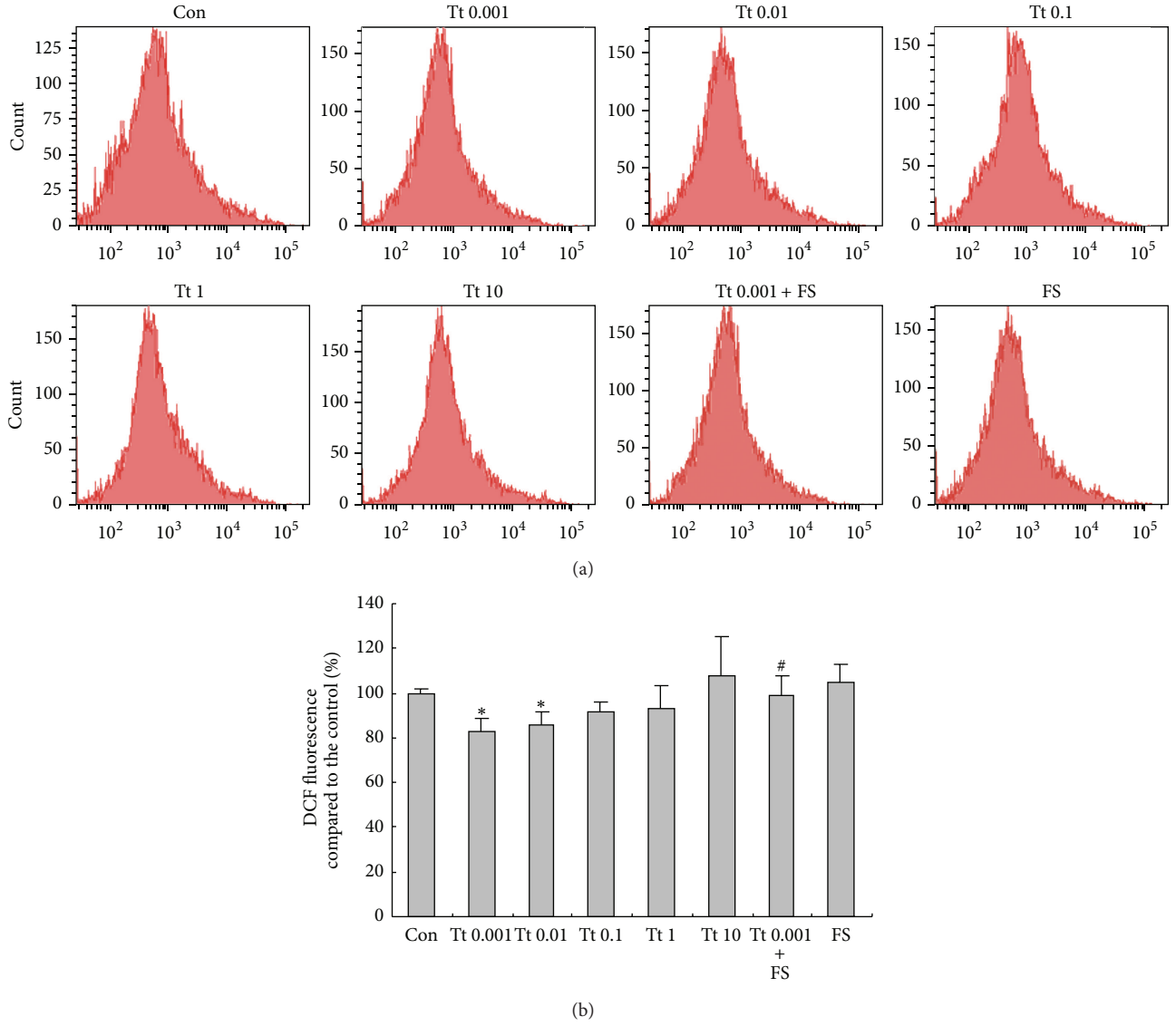

Figure 2: Effects of tryptase (Tt) on the generation of reactive oxygen species (ROS) from astrocytes. Astrocytes were exposed to different concentrations of tryptase (0.001, 0.01, 0.1, 1, and 10 μg/mL) in the presence or absence of FSLLRY-NH2 (FS, 400 μM), an antagonist of tryptase at 37°C for 2 h. ROS was presented as changes of DCF fluorescence. Data are presented as the mean ± SEM analyzed from three independent sets of samples. $^{*}P < 0.05$ versus control group (Con), $^{\#}P < 0.05$ versus corresponding tryptase treatment group.

(20 μM), PD98059 (20 μM), SP600125 (20 μM), and LY294002 (20 μM) alone did not have impacts on the secretion of IL-6 and TNF-α from astrocytes. tryptase (1 μg/mL) induced IL-6 increase was reversed by LY294002, but not by inhibitors of MAPKs (SB203580, PD98059, SP600125) (Figure 4(a)). However, tryptase (1 μg/mL) induced TNF-α increase was reversed by SB203580, PD98059, SP600125, and partially by LY294002 (Figure 4(b)), indicating that PI3K/AKT signaling pathway contributes to the secretion of TNF-α and IL-6 induced by tryptase, respectively, whereas MAPKs signaling pathway is involved in the tryptase induced secretion of TNF-α, but not IL-6.

3.5. Tryptase Activated MAPKs and PI3K/AKT in Primary Cultured Astrocytes via PAR-2. In order to further understand the actions of tryptase on astrocytes, we examined the effects of tryptase on phosphorylation of cell signaling molecules. Tryptase at 1 μg/mL activated p38 MAPK, JNK, ERK (p44/42), and AKT, which was confirmed by increased phosphorylation of tyrosine residues of these kinases as determined by Western blot analysis. The time course experiments showed that treatment with tryptase (1 μg/mL) led to a rapid and transient phosphorylation of MAPKs and AKT with the peak levels of phospho-p38, phospho-JNK, and phosphor-ERK (p44/42) occurring at 30 min, and phospho-AKT at 60 min, respectively (Figure 5(a)). Astrocytes were pretreated with PAR-2 antagonist FS (200 and 400 μM) for 30 min and then exposed to tryptase (1 μg/mL) for another 30 min or 60 min for detecting the phosphorylation of MAPKs and AKT, respectively. PAR-2

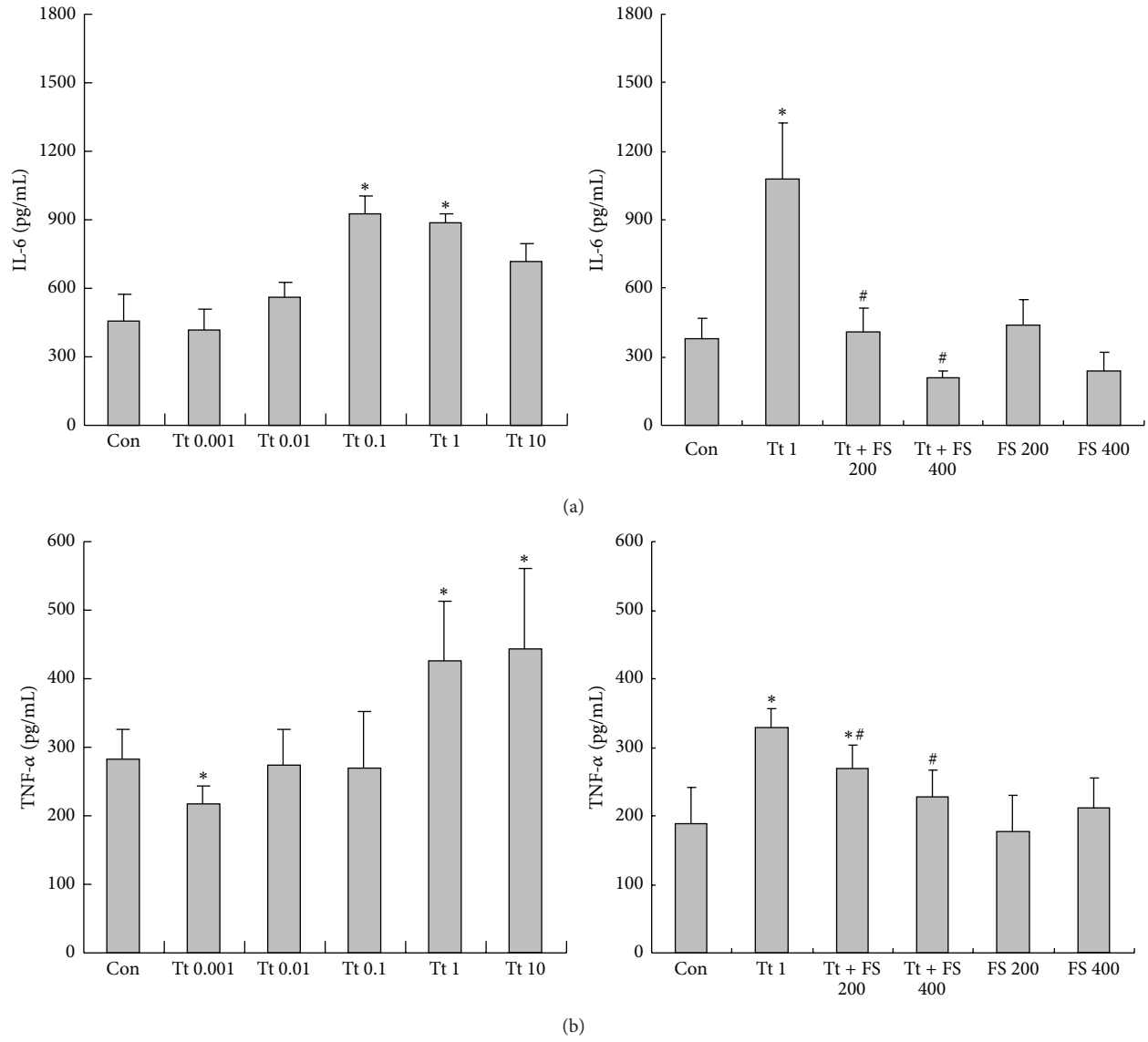

Figure 3: Effects of tryptase (Tt) on the secretion of IL-6 (a) and TNF-α (b) from astrocytes. Astrocytes were exposed to different concentrations of tryptase (0.001, 0.01, 0.1, 1, and 10 μg/mL) in the presence or absence of FSLLRY-NH2 (FS, 200 or 400 μM), an antagonist of tryptase at 37°C for 24 h before culture supernatant being collected. Data are presented as the mean ± SEM of four independent experiments. $^{*}P < 0.05$ versus control groups (Con), $^{\#}P < 0.05$ versus corresponding tryptase treatment groups.

antagonist FS alleviated tryptase-induced MAPKs activation but itself alone had no effect on the phosphorylation of MAPKs and AKT in astrocytes (Figure 5(b)), indicating that activation of PAR-2 by tryptase might be responsible for the activation of MAPKs and PI3K/AKT.

3.6. Tryptase Enhanced TGF-β and CNTF Expression in Primary Cultured Astrocytes. The time course study (incubation with 1 μg/mL tryptase for 0, 30, 60, 120, 240 min) showed that tryptase significantly promoted TGF-β expression in astrocytes, which began at 2 h and lasted at least until 4 h. However, an increase of CNTF expression induced by tryptase (1 μg/mL) initiated rapidly at 1 h following incubation and lasted at least until 4 h (Figure 6). The enhancement in TGF-β and CNTF levels indicates that tryptase can induce endogenous production of TGF-β and CNTF, which probably contributes to neuroprotection.

4. Discussion

Astrocytes, microglia, and endothelial cells are involved in the intracerebral immune response where they act, in part, by secreting cytokines, chemokines, neurotrophic or neurotoxic factors [27]. Among them, astrocytes play an essential role in neuronal life-support and contribute to the BBB. They also act as neuroprotectors by secreting neurotrophins and release potentially toxic inflammatory molecules [28]. So in the present work, we put our focus on astrocytes with an emphasis on its potential role in the cytokine production involved in neuroimmune processes.

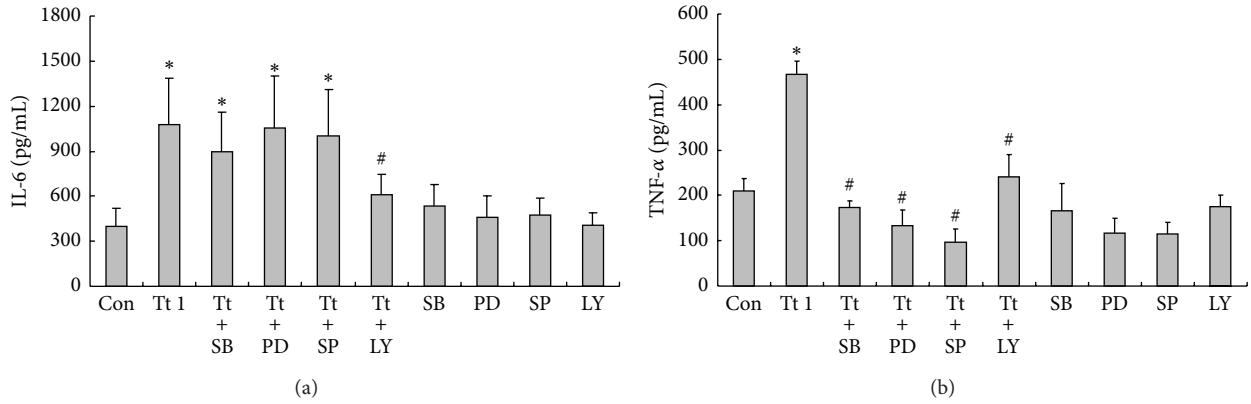

Figure 4: Effects of inhibitors of cell signaling pathways on tryptase (Tt)-induced secretion of IL-6 (a) and TNF-α (b) from astrocytes. Astrocytes were exposed to 1 μg/mL of tryptase in the presence or absence of SB203580 (SB, 20 μM), PD98059 (PD, 20 μM), SP600125 (SP, 20 μM), and LY294002 (LY, 20 μM) at 37°C for 24 h before culture supernatant being collected. Data are presented as the mean ± SEM of four independent experiments. $^{*}P < 0.05$ versus control groups (Con), $^{\#}P < 0.05$ versus corresponding tryptase treatment groups.

In this study, we first found that tryptase is able to regulate cytokines release from astrocytes without affecting astrocytic viability and proliferation via PAR-2-MAPKs or PAR-2-PI3K/AKT signaling pathway, revealing a novel profile of PAR-2 as a new target in the regulation of astrocytic function.

Tryptase, a major secretory protein of human mast cells, is an endogenous peptide which specifically activates PAR-2. Incubation with tryptase at low concentrations (0.001 and 0.01 μg/mL) modestly inhibited the intracellular levels of ROS in astrocytes, indicating this protease is a potent factor to modulate astrocytes-derived ROS. The specific antagonist peptide of PAR-2 FS diminished the effect of tryptase on ROS generation, implying that the inhibition of the intracellular ROS by tryptase might at least partially be responsible for the activation of PAR-2. In the brain, ROS exerts a key role in normal physiological functions and neuroimmune responses [26, 29], and astrocytes are the important sources of ROS. Imbalance in the level of ROS has been shown to be a causative factor in numerous pathologies such as ischemia/reperfusion injury and degenerative diseases [29, 30]. Considerable amount of evidence suggests oxidative stress induced by astrocytes-derived ROS is a crucial contributor to neurodegeneration [31, 32]. Our current observation implicates that moderate stimulation of PAR-2 existed in astrocytes might regulate astrocytes-derived ROS and supplies a new option for the management of CNS functions.

IL-6 is a pleiotropic cytokine involved in the regulation of inflammatory and immunological responses, acute phase protein production, and hematopoiesis [33]. There is increasing evidence supporting a role for the IL-6 receptor family in CNS development, as well as during neurodegeneration and regeneration [34]. Like many cytokines, IL-6 may have distinct physiological effects at different concentrations and in different biological contexts [35]. Like IL-6, TNF-α now appears also to play an important role in neural plasticity and neurorepair [36–38] in addition to its well established function as a proinflammatory cytokine. Here we presented data investigating the consequence of PAR-2 activation by various concentrations of tryptase on IL-6 and TNF-α secretion from astrocytes. We found that tryptase administration produced a concentration dependent increase in IL-6 and TNF-α secretion with a minimum effective dose of 0.1 μg/mL and 1 μg/mL, respectively, suggesting that the stimulation of IL-6 generation by tryptase is more sensitive than that of TNF-α in astrocytes, which is distinct from the data obtained from microglia [39]. Since neuroinflammation could generate an environment detrimental for repair, alternatively it could also create an environment permissive for neurorepair [40], our findings implicate that mast cell tryptase is able to regulate the activity of astrocytes and the levels of neuroinflammatory proteins released from astrocytes such as IL-6 and TNF-α so as to modulate the balance between neuroinflammation and neurorepair [33].

Moreover, tryptase-induced IL-6 and TNF-α release from astrocytes appeared to rely on the activation of PAR-2 as a specific antagonist peptide of PAR-2 FS was able to block the action of tryptase on astrocytes. PAR-2 was identified to be expressed abundantly on astrocytes [6], PAR-2 activation in astrocytes has been demonstrated to play a key modulatory role in diverse pathological conditions [15, 41, 42], but the underlying mechanisms remain to be clarified. The intracellular downstream MAPKs have been demonstrated to be involved in the regulation of astrocytic function including the production of neuroinflammatory factors in astrocytes. Indeed, our results showed that the intracellular signaling mechanisms that mediate tryptase-induced TNF-α release rather than IL-6 release from astrocytes are p38, JNK, and ERK dependent, as SB203580, PD98059, and SP600125 all significantly eliminated TNF-α release. As well, tryptase-induced TNF-α increase was partly due to PI3K/AKT signaling. Similarly, PI3K/AKT signaling is also responsible for the enhanced production of IL-6, which is consistent with the finding previously reported [43]. Thus, our data provide new evidence that PI3K/AKT signaling pathway contributes to the

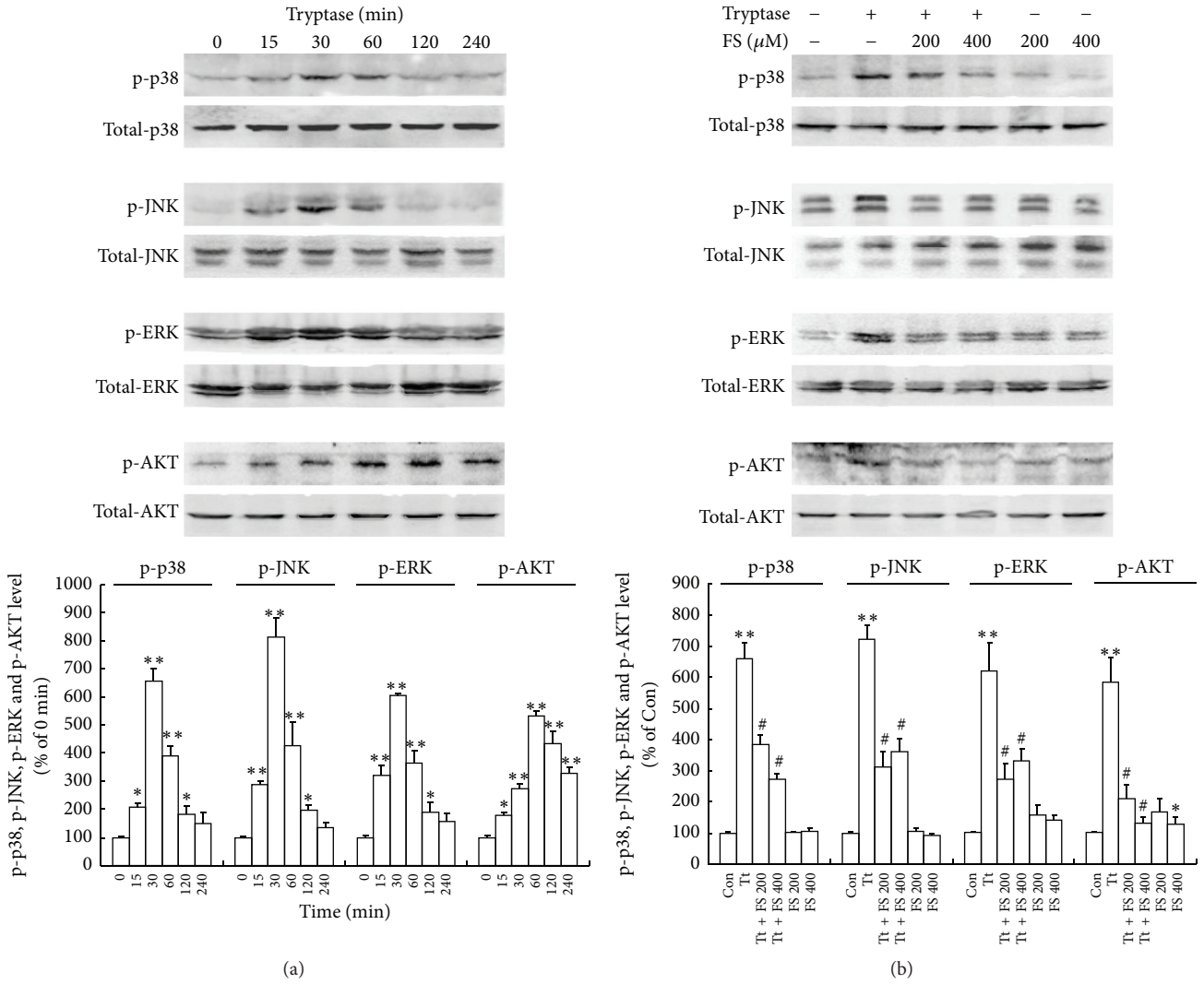

Figure 5: Effects of tryptase (Tt) on the activation of MAPKs and PI3K/AKT. (a) Time courses of tryptase activated p38, JNK, ERK, and AKT, which was assessed by increased phosphorylation of tyrosine residues of these kinases. Astrocytes were incubated with 1 μg/mL of tryptase for the indicated periods (0, 15, 30, 60, 120, and 240 min). (b) FSLLRY-NH2 (FS, 200 or 400 μM) alleviated tryptase (1 μg/mL) induced MAPKs and PI3K/AKT activation. Astrocytes were incubated with 1 μg/mL of tryptase in the presence or absence of FS, an antagonist of tryptase at 37°C for 30 min. Activated p38 (p-p38), JNK/SAPK (p-JNK), ERK (p-ERK), and AKT (p-AKT) species were detected by immunoblot analysis with antibodies specific for the phosphorylated forms of each kinase. The amount of protein loaded in each lane was confirmed by measuring the amount of p38, JNK, ERK, and AKT reacted to the antibody against the unphosphorylated form of each kinase. This is a representative experiment independently performed three times. $^{**}P < 0.01$, $^{*}P < 0.05$ versus control groups (Con), $^{\#}P < 0.05$ versus corresponding tryptase treatment groups.

secretion of TNF-α and IL-6 induced by tryptase, whereas MAPKs signaling pathway is involved in the tryptase induced secretion of TNF-α, but not IL-6.

In addition, since TGF-β and CNTF are potent neural cytokines with very low expression predominantly by astrocytes [44] and both cytokines play very important roles in the modulation of CNS function including neuroinflammation and neurorepair, the levels of TGF-β and CNTF expressed in astrocytes were determined in our study. We found that tryptase administration significantly upregulated TGF-β and CNTF expression in astrocytes initiated at 2 h and 1 h, respectively, implying that tryptase is as well potent to increase endogenous levels of TGF-β and CNTF which probably contribute to neuroprotection. CNTF is almost exclusively produced in the nervous system and can rescue various types of adult CNS neurons in disease models [45–47]. TGF-β performs a critical function in nervous system development and repair [48–50]. Both of them are closely implicated in neurodevelopment and neurorepair. Our results not only reveal tryptase as a potent factor to regulate TGF-β and CNTF production in astrocytes but may also provide a novel therapeutic option to neurological disorders. Obviously, more detailed work is required to address the issue further.

In conclusion, to our knowledge, this is the first study to demonstrate the ability of mast cell tryptase in modulation of astrocytic activation and astrocytes-derived cytokine

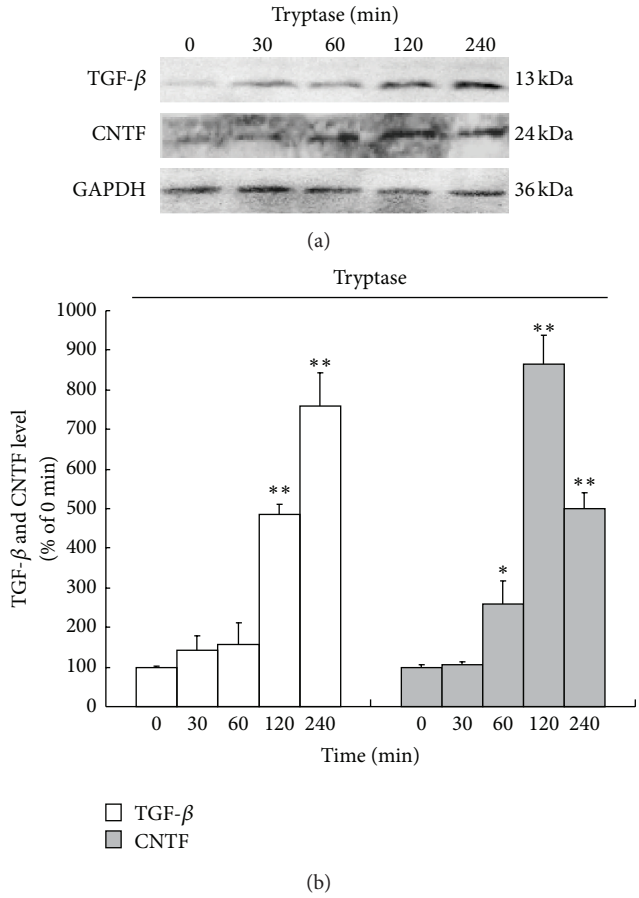

Figure 6: Time courses of tryptase (Tt) on the expression of transforming growth factor-β (TGF-β) and ciliary neurotrophic factor (CNTF) in astrocytes. (a) Tryptase at 1 μg/mL was incubated with astrocytes at 37°C for 0, 30, 60, 120, and 240 min, respectively. Protein levels were detected by immunoblot analysis with specific antibodies. Western blots at the top of each panel are from a typical experiment. (b) Bar graphs are the quantified results expressed as mean ± SEM of TGF-β and CNTF levels from three independent experiments. $^{**}P < 0.01$, $^{*}P < 0.05$ versus 0 min.

production via PAR-2. The implicated signaling mechanisms that mediate the actions of tryptase might be PAR-2-MAPKs or PAR-2-PI3K/AKT pathways, providing a novel profile of PAR-2 as a new target in the regulation of astrocytic function.

Conflict of Interests

The authors report no financial or other conflict of interests relevant to the subject of this paper.

Authors' Contribution

X. Zeng and S. Zhang contributed equally to this work.

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Research Article

Local Overexpression of Interleukin-11 in the Central Nervous System Limits Demyelination and Enhances Remyelination

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Demyelination is one of the pathological hallmarks of multiple sclerosis (MS). To date, no therapy is available which directly potentiates endogenous remyelination. Interleukin-11 (IL-11), a member of the gp130 family of cytokines, is upregulated in MS lesions. Systemic IL-11 treatment was shown to ameliorate clinical symptoms in experimental autoimmune encephalomyelitis (EAE), an animal model of MS. IL-11 modulates immune cells and protects oligodendrocytes *in vitro*. In this study, the cuprizone-induced demyelination mouse model was used to elucidate effects of IL-11 on de- and remyelination, independent of the immune response. Prophylactic-lentiviral- (LV-) mediated overexpression of IL-11 in mouse brain significantly limited acute demyelination, which was accompanied with the preservation of $CC1^+$ mature oligodendrocytes (OLs) and a decrease in microglial activation ($Mac\text{-}2^+$). We further demonstrated that IL-11 directly reduces myelin phagocytosis *in vitro*. When IL-11 expressing LV was therapeutically applied in animals with extensive demyelination, a significant enhancement of remyelination was observed as demonstrated by Luxol Fast Blue staining and electron microscopy imaging. Our results indicate that IL-11 promotes maturation of $NG2^+$ OPCs into myelinating $CC1^+$ OLs and may thus explain the enhanced remyelination. Overall, we demonstrate that IL-11 is of therapeutic interest for MS and other demyelinating diseases by limiting demyelination and promoting remyelination.

1. Introduction

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS) that develops in genetically predisposed individuals where tolerance to self-antigens is broken down [1]. One of the pathological hallmarks of MS is loss of the nerve-insulating myelin sheath, which contributes to classical symptoms observed in individuals with MS including loss of sensation, motor, autonomic and neurocognitive functions [2, 3]. Infiltrated macrophages and resident microglia together with activated autoreactive lymphocytes orchestrate immune-mediated demyelination and concomitant axonal degeneration (reviewed in McFarland and Martin 2007) [4]. In addition to the release of cytotoxic cytokines or soluble toxic mediators [5, 6], microglia and infiltrated macrophages phagocytose and degrade myelin and are considered the main effector cells in MS. While there is evidence for endogenous remyelination in MS lesions, it fails in the majority of patients [7, 8].

Current treatments of MS aim to limit inflammation, thereby reducing the incidence of new lesions and clinical relapses. These approaches, so far, demonstrated little direct effects on regeneration and remyelination [9]. Endogenous remyelination is achieved through the rapid amplification of oligodendrocyte progenitor cells (OPCs) in demyelinated areas and their subsequent maturation into myelinating oligodendrocytes (OLs). However, remyelination is often ineffective in MS. While OPCs are still found in chronic MS

lesions, they fail to differentiate into oligodendrocytes [10]. The reasons for this phenomenon are likely determined by intrinsic changes within the oligodendrocyte precursor cell population in combination with the specific inhibitory cues present in multiple sclerosis lesions (for detailed review, see Kotter et al. 2011 [11]). Factors that increase the survival, proliferation, migration, and differentiation of OPCs can favorably tweak the dysregulated signaling environment and thus promote remyelination. In this regard, Interleukin-11 (IL-11), a member of the gp130 cytokine family [12] with its well-established immunomodulatory and neuroprotective effects, can be considered as a potential therapeutic candidate. Like several other gp130 signaling cytokines, including leukemia inhibitory factor (LIF) and Oncostatin M (OsM), IL-11 is upregulated in MS lesions [13]. As LIF was shown to limit autoimmune-mediated demyelination in EAE [14], the upregulation of IL-11 in MS lesions could indicate its putative role in lesion regulation. IL-11 was reported to promote Th2 polarization of $CD4^+$ T cells and reduce the production of toxic mediators by macrophages [15–17]. Moreover, IL-11 enhances the survival of OPCs by inducing STAT3-mediated antiapoptotic effect [13, 18]. While IL-11Rα knockout mice show increased clinical symptoms of EAE, systemic treatment of wild-type EAE animals reduced disease, indicating that IL-11 is able to regulate autoimmune demyelination [19].

In this study, we used the cuprizone mouse model to characterize direct effects of IL-11 on de- and remyelination, independent of autoimmune modulation. Mice fed with cuprizone exhibit a reproducible and reversible demyelination accompanied by proliferation of OPCs and microglia in the midline region of corpus callosum. When returned to normal chow, spontaneous remyelination occurs as a result of OPC maturation to myelinating OLs [20, 21]. Our study reveals that CNS-targeted overexpression of IL-11 limits demyelination on one hand and in addition accelerates remyelination of demyelinated regions making it an interesting therapeutic target that directly enhances regeneration.

2. Materials and Methods

2.1. Development of Lentiviral Vectors and Analysis of Transgene Expression. Lentiviral vectors (LVs) are a powerful tool to obtain stable transgene expression in the central nervous system [22, 23]. HIV-1 derived, second generation LV vectors, encoding enhanced fluorescent protein (eGFP), and murine IL-11 were produced using triple transient transfection of 293T cells, as previously described [14]. Briefly, 293T cells were transfected with a packaging plasmid, a plasmid encoding the glycoprotein G of vesicular stomatitis virus and a transfer plasmid encoding murine IL-11 (NCBI Reference Sequence: NM_008350) or eGFP under the control of cytomegalovirus promoter. Vector particles in the supernatant were concentrated using Vivaspin 15 columns (Vivascience, Hannover, Germany), aliquoted and stored at −80°C. p24 antigen content was determined by HIV-1 p24 Core Profile ELISA (DuPont, Dreieich, Germany). To confirm transgene expression *in vitro*, HEK 293T cells were transduced with IL-11-LV stock solution corresponding to $1.045 \pm 0.035 \times 10^7$ pg of p24/mL, as described previously [14]. Expression of IL-11 protein was determined 72 h after transduction, by immunocytochemistry using monoclonal anti-mouse IL-11 antibody (2 μg/mL) (R and D Systems; MAB418). Secretion of IL-11 in the supernatant of transduced 293T cells was measured by ELISA (R and D systems; DY418) 48 and 72 h after transduction. For the *in vivo* validation of transgene expression, mice were stereotactically injected with 4 μL of IL-11-LV stock solution. After 2 weeks, their brains were isolated and processed for IL-11 immunostaining.

2.2. Stereotactic Administration. Mice were anesthetized with ketamine (80 mg/kg i.p.) and medetomidine (6 mg/kg i.p.) and fixed in the stereotactic apparatus (Stoelting, IL, USA). The skull was exposed through a small midline incision in the scalp. A hole was drilled in the skull at the chosen coordinates (in reference to bregma), namely, anterioposterior, −0.5 mm; lateral, −2.0 mm; and dorsoventral, −2.0 mm [24] (Figure 1 in Supplementary Material available online at http://dx.doi.org/10.1155/2013/685317). A 30 gauge needle fitted with 10 μL hamilton syringe was inserted in the brain. After acclimatizing the needle for 5 min, 4 μL of respective treatments (Phosphate buffer saline (PBS)/eGFP-LV/IL-11-LV) was administered at the rate of 0.25 μL/min with the help of "Quintessential" stereotactic injector (Stoelting, IL, USA). The needle was kept in place for an additional 5 min before it was slowly retracted. The scalp was sutured and disinfected. Atipamezole (1 mg/kg i.p.) was administered and mice were allowed to recover at 37°C for 24 h. All procedures were performed under aseptic conditions. All animal experiments were approved by the Hasselt University ethics committee.

2.3. Cuprizone Treatment and Experimental Groups. Male, adult C57BL/6J mice aged 6 to 8 weeks were purchased from Harlan. The mice were housed in a 12 h light/dark cycle at an ambient temperature and had free access to food and water. Cuprizone (Sigma Aldrich; C9012) was homogenously mixed (0.2% w/w) with powdered rodent chow and was changed daily. Two treatment regimens were chosen: (1) prophylactic overexpression of IL-11 to evaluate its effect on acute demyelination and (2) therapeutic overexpression of IL-11 (after demyelination) to evaluate its effect on remyelination. The experimental design of the two approaches is graphically illustrated in Figures 1(a) and 4(a), respectively. To induce acute demyelination, mice were fed with cuprizone diet for 5 weeks. To allow spontaneous remyelination, mice were switched to standard pelleted chow for two weeks [20, 21]. In both experiments, mice were randomly divided into three experimental groups, namely, (1) PBS treated, (2) eGFP-LV treated, and (3) IL-11-LV treated. Healthy animals were used as a control for immunohistochemistry and histology.

2.4. Histology and Immunohistochemistry. After completion of both study protocols, brains were excised, embedded in Tissue-Tek, and snap-frozen in liquid nitrogen. Frozen sections (10 μm thick) were cut with a microtome (Leica Microsystems, Wetzelar, Germany). The myelinated area was assessed by luxol fast blue staining. To do so, acetone-fixed

mouse brain coronal sections were incubated at 56°C for 16 h in luxol fast blue solution. Sections were then differentiated in 0.5% lithium carbonate solution for 45 s and counterstained with cresyl violet. Immunohistochemistry was performed using primary antibodies, namely, rat anti-IL-11 (5 μg/mL; R and D systems; MAB 418), rabbit anti-NG2 (1 : 200; Millipore; AB5320), rabbit anti-Iba-1 (1 : 350; Wako chemicals; 019-19741), mouse anti-Mac-2 (1 : 250; ATCC; clone M3/38.1.2.8 HL.2), and mouse anti-CC1 (2 μg/mL; Calbiochem; OP80). For all immunostainings (except CC1), sections were fixed in ice-cold acetone for 10 min and air-dried for 30 min. For CC1 immunostaining, sections were fixed in ice-cold 4% paraformaldehyde buffer (pH = 7.3) for 10 min and then kept in boiling 0.01 M citrate buffer for 30 min for antigen retrieval. Cryosections were blocked with 10% goat serum in 0.05% PBS-Tween20 (PBS-T) for 1 h and incubated with respective concentrations of primary antibody diluted in blocking buffer, for 3 h at room temperature. The sections were then incubated with goat anti-species secondary antibodies conjugated with Alexa-488, 555 or 568 (Invitrogen) for 2 h at room temperature. DAPI was used as nuclear stain. All sections from one study were treated identically side by side.

2.5. Transmission Electron Microscopy (TEM). The sample preparation for TEM was performed as previously described [25] with minor modifications. Briefly, mice were transcardially perfused with ice-cold 2% glutaraldehyde in 0.05 M cacodylate buffer (pH = 7.3) under deep pentobarbitone anesthesia. A coronal brain block (1 mm thick) within the anterioposterior coordinates from −0.3 to −1.5 mm was cut in the midsagittal plane. Subsequently, tissue was postfixed in 2% osmium tetroxide for 1 h, stained with 2% uranyl acetate in 10% acetone for 20 min, dehydrated through graded concentrations of acetone, and embedded in epoxy resin (Araldite). Semithin sections (0.5 μm) were stained with a solution of thionin and methylene blue (0.1% aqueous solution) for light microscopic examination to delineate the region of interest. Ultrathin sections (0.06 μm) from selected tissue blocks were mounted on 0.7% formvar-coated grids, contrasted with uranyl-acetate followed by lead citrate, and examined on a Philips EM 208 transmission electron microscope (Philips, Eindhoven, The Netherlands) operated at 80 kV.

2.6. Quantification. For histological quantifications, images were acquired using a Nikon eclipse 80i microscope (Nikon corporations, Japan). Adobe Photoshop CS4 was used to adjust image contrast and brightness (all images were adjusted equally within an experiment). A mosaic of images of the corpus callosum was prepared for each section. For each mouse, at least three coronal sections 150 μm apart were used for analysis. These sections were obtained in the splenium and dorsal hippocampal commissure regions [26], that is, between the anterioposterior coordinates of −0.3 to −1.5 mm with respect to bregma. Quantification was performed in the region of interest (ROI) (Supplementary Figure 1) spanning the corpus callosum from the contralateral to the ipsilateral horn using ImageJ software (1.44p; http://imageJ.nih.gov/ij). The myelinated area in the corpus callosum of luxol fast blue-stained sections was quantified using a manually set threshold, equivalent to all images. Fluorescence-based morphometric cell counting was performed manually. Individual cells were counted based on the presence of nuclei. The number of myelinated fibres and G-ratio was quantified in the electron micrographs of the cross-section of the midsagittal corpus callosum (at least three mice/group). G-ratio was calculated as axon diameter/fiber diameter. Individual axons were categorized based on their individual G-ratio as spared (G-ratio < 0.74), remyelinating (0.74 ≤ G-ratio > 0.90), and demyelinated (G-ratio ≥ 0.90) axons. Images from nonserial ultrathin sections were captured at a magnification of 11kX, and five representative images were selected per mouse by a blinded observer for evaluation using ImageJ software.

2.7. Myelin Phagocytosis Assay. To analyze myelin phagocytosis, the BV2 microglial cell line was plated in 24-well plates (7.5×10^4 cells/well) and incubated for 2 h at 37°C and 5% CO_2. Cells were subsequently treated for 12 h with logarithmically increasing concentrations (1, 3, 10, and 30 ng/mL) of recombinant mouse (rm) IL-11 (R and D systems; 418-ML). To the control cells, no cytokine was added. Next, the medium was changed and a myelin phagocytosis assay was performed as described previously [27]. In short, DiI-labeled myelin (50 μg /well) was added to the BV2 cultures and incubated for 90 min at 37°C and 5% CO_2. Noningested and nonbound myelin was removed by washing the plates with ice-cold PBS. Cells were detached with 5 mM EDTA in PBS for 15 min. The amount of myelin phagocytosed was determined by measuring the cellular DiI fluorescence intensity using a FACScalibur flow cytometer (BD Biosciences, Erembodegem, Belgium).

2.8. Statistical Methods. Data is analyzed using Graph-Pad prism (GraphPad software Inc, USA). All results are expressed as mean ± SEM. Statistical significance is assessed by one-way ANOVA followed by Bonferroni's multiple comparison test. P values smaller than 0.05 are considered significant.

3. Results

3.1. Prophylactic Overexpression of IL-11 Limits Cuprizone-Induced Demyelination. First, the murine IL-11 encoding lentiviral vector (IL-11-LV) was verified for its expression *in vitro* as well as *in vivo* (Supplementary Figure 2). Consequently, the effect of prophylactic overexpression of IL-11 on acute demyelination was investigated. To allow stable overexpression, lentiviral vectors were stereotactically administered in the brain of healthy mice, 2 weeks prior to starting the cuprizone diet. PBS and eGFP-LV treatments were used as controls (Figure 1(a)). After 5 weeks of cuprizone diet, the corpus callosum appears hypercellular and edematous. Luxol fast blue staining revealed significant demyelination in the corpus callosum (Figure 1(b)). A significant ($P < 0.001$) decrease in the myelinated area was observed in the PBS and eGFP-LV treated cuprizone groups (0.4209 ± 0.01299 and 0.4884 ± 0.01548 mm^2, resp.) as compared to healthy mice (0.7160 ± 0.01803 mm^2). Prophylactic overexpression of

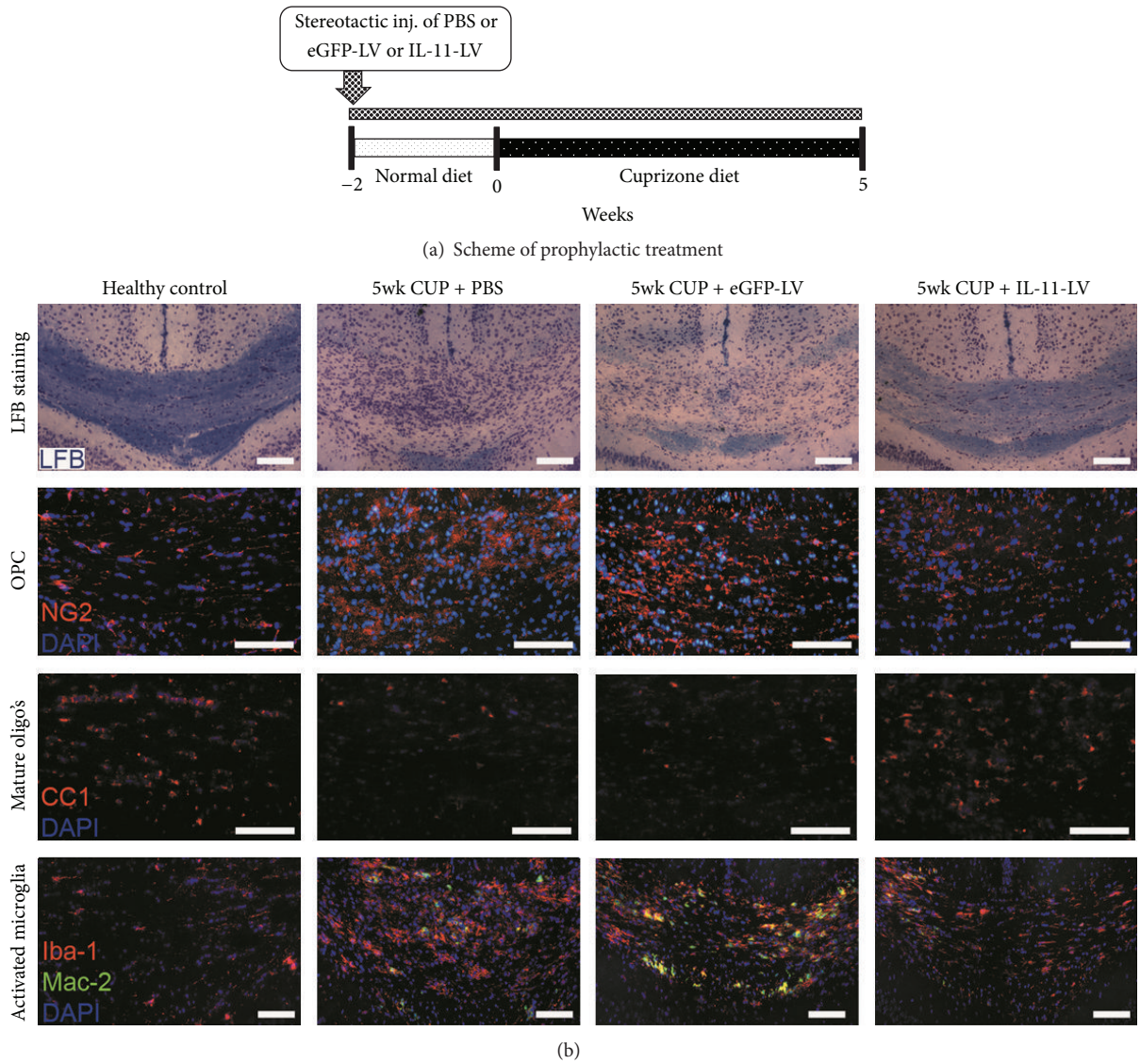

Figure 1: Scheme and effect of prophylactic overexpression of IL-11 on acute demyelination. (a) Murine IL-11-LV was administered stereotactically, 2 weeks prior to starting of the cuprizone (CUP) diet. PBS and eGFP-LV treatments were included as controls. After 5 weeks of cuprizone diet, animals were sacrificed and processed for immunohistochemistry. Panel (b) shows representative images of the midline corpus callosum in coronal brain sections of all groups, depicting demyelination (LFB), density of OPCs (NG2), OLs (CC1), and activated microglia (Mac-2/Iba-1). The images are chosen between the anterioposterior coordinates from −0.3 to −1.5 mm in reference to bregma. Scale bar: 200 μM.

IL-11 prevented cuprizone-induced demyelination by 60% as evident by a significantly larger ($P < 0.001$) myelinated area (0.6204 ± 0.02318 mm^2) compared with the eGFP-LV treated cuprizone group (Figure 2(a)). Thus, local expression of IL-11 limits acute demyelination in the CNS.

3.2. Prophylactic Overexpression of IL-11 Limits Mature OLs Loss and Subsequent OPCs Proliferation. Apoptosis of mature OLs has been implicated as the primary cause of demyelination in the cuprizone model [28–30]. After 5 weeks of cuprizone diet, the density of CC1$^+$ mature OLs dropped significantly ($P < 0.001$) from 453.7 ± 36.61/mm^2 in healthy mice to 218.8 ± 9.95 and 184.3 ± 13.88/mm^2 in PBS and eGFP-LV treated groups, respectively. Prophylactic overexpression of IL-11 significantly ($P < 0.05$) reduced cuprizone-induced loss of OLs by 46% as evidenced by a higher density (308.0 ± 28.66/mm^2) of these cells in corpus callosum (Figures 1(b) and 2(b)). In line with the previous reports, a 4-5-fold increase of NG2$^+$ OPCs was observed after 5 weeks of cuprizone diet, reflecting proliferation of OPC in response to depletion of mature OLs [21, 31]. The density of OPCs significantly ($P < 0.05$) increased from 134.1 ± 6.14/mm^2 in healthy mice to 529.4 ± 57.31 and 446.5 ± 74.33/mm^2 in PBS and eGFP-LV treated cuprizone groups, respectively.

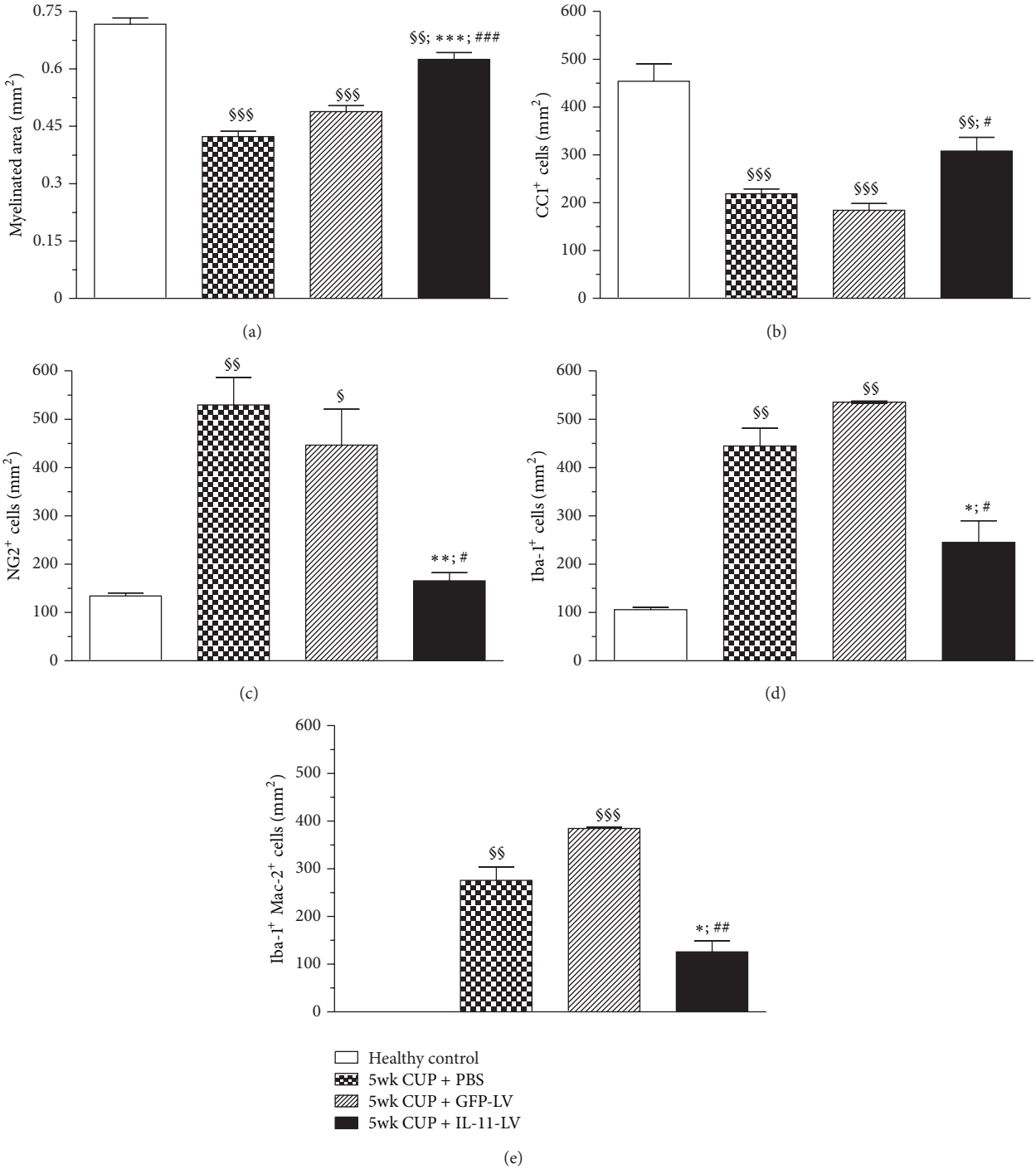

Figure 2: Quantification of the effect of prophylactic overexpression of IL-11 on demyelination, OPCs, OLs, and microglia. Quantitative analysis of coronal brain sections revealed that IL-11 (i) *limits demyelination*, as evident by a larger luxol fast blue-stained myelinated area (a), (ii) *prevents the degeneration of mature OLs and limits proliferation of OPC's* as demonstrated by immunostaining with CC1 (b) and NG2 (c), and (iii) *limited microgliosis* (d) *and activation of microglia* (e). Data is expressed as mean ± SEM (n = 5 animals per group). Statistical significance is analyzed by one-way ANOVA followed by Bonferroni's multiple comparison test. $^{\S}P < 0.05$, $^{\S\S}P < 0.01$, $^{\S\S\S}P < 0.001$ as compared to healthy control; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to 5 wk CUP + PBS group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared to 5 wk CUP + eGFP-LV group. Quantification was performed in the corpus callosum of at least three coronal brain sections per mouse.

Prophylactic overexpression of IL-11 significantly prevented ($P < 0.05$) the proliferation of $NG2^+$ OPCs as their density ($165.8 \pm 16.56/mm^2$) was almost equivalent to that of normal healthy values (Figures 1(b) and 2(c)). Together these data show that IL-11 prevents the cuprizone-induced OLs cell death, thereby limiting the proliferation of OPCs in the demyelinating regions.

3.3. Prophylactic Overexpression of IL-11 Prevents Microglial Proliferation and Activation. Extensive microgliosis is observed in the region of cuprizone-induced demyelination. In line with earlier findings, we observed a 4-5-fold increase in the density of Iba-1^+ microglia in the corpus callosum after 5 weeks of cuprizone diet. The density of microglia grew significantly ($P < 0.01$) from $105.9 \pm 4.97/mm^2$ in the healthy control group to 444.4 ± 37.01 and $535.1 \pm 2.212/mm^2$ in PBS and eGFP-LV treated groups, respectively. Prophylactic overexpression of IL-11 significantly reduced ($P < 0.05$) the proliferation of microglia by 63%, and its density decreased to $245.2 \pm 44.76/mm^2$ (Figures 1(b) and 2(d)). Moreover, prophylactic overexpression of IL-11 limited the activation of microglia as shown by the density of Iba-1^+Mac-2^+ cells ($125.7 \pm 22.85/mm^2$), which was observed to be significantly lower ($P < 0.05$) as compared with the PBS ($275.6 \pm 27.86/mm^2$) and eGFP-LV ($384.5 \pm 2.38/mm^2$) treated groups. Thus, local expression of IL-11 in the CNS limits microglia activation during demyelination.

3.4. IL-11 Inhibits Myelin Phagocytosis by BV2 Cells In Vitro. To further dissect the direct effect of IL-11 on microglial activation in the context of myelin breakdown, we investigate the effect of IL-11 on myelin phagocytosis by BV2 (microglial) cells line *in vitro*. Pretreatment of BV2 cells with rmIL-11 (1, 3, 10, and 30 ng/mL) for 12 h dose dependently reduced ($P < 0.001$) subsequent phagocytosis of DiI-labeled myelin (Figure 3).

3.5. Therapeutic Overexpression of IL-11 Enhances Remyelination. We next sought to investigate the direct effect of IL-11 on endogenous remyelination. Therefore, animals were kept on a cuprizone diet for 5 weeks to induce complete demyelination. One week prior to that, IL-11-LV or respective controls were administered to allow for a sustained and maximal overexpression. Animals were then allowed to remyelinate spontaneously by switching to standard diet for 2 weeks (Figure 4(a)). Therapeutic overexpression of IL-11 showed an accelerated endogenous remyelination in the corpus callosum. As shown by luxol fast blue staining, the myelinated area in IL-11-LV treated group ($0.5311 \pm 0.01893\ mm^2$) was found to be significantly ($P < 0.01$) higher as compared to the PBS treated group ($0.4036 \pm 0.01793\ mm^2$). A trend of increased remyelination was observed as compared to the eGFP-LV treated group ($0.4544 \pm 0.01643\ mm^2$) although not statistically significant. Therapeutic overexpression of IL-11 was able to fasten spontaneous remyelination, but not up to the extent of the healthy control group ($0.6540 \pm 0.02711\ mm^2$). To further verify whether IL-11 directly affects remyelination, we analyzed ultra-thin cross sections of the corpus callosum by electron microscopy. The G-ratio (ratio of the inner axonal diameter to the total fiber diameter) is considered to be the functional and structural index of optimal axonal myelination [32]. In the IL-11-LV treated group, the G-ratio (0.7452 ± 0.012) of myelinated axons was significantly ($P < 0.05$) lower as compared to that in the PBS and eGFP-LV treated groups (0.8146 ± 0.01; 0.8066 ± 0.02), respectively. Moreover, the G-ratio of the IL-11-LV treated group was higher than that in the healthy control group (0.7238 ± 0.006) indicating that the myelin sheath is newly formed and therefore relatively thin (Figure 5(b)). The mean axonal diameter was not significantly different among the groups (Figure 5(c)). Four weeks of cuprizone diet led to a significant decrease ($P < 0.001$) in the density of myelinated axons ($2.09 \pm 0.53 \times 10^5/mm^2$) as compared to the healthy control group ($7.69 \pm 0.95 \times 10^5/mm^2$). After two weeks of endogenous remyelination, the number of myelinated axons increased in the PBS, eGFP-LV, and IL-11-LV treated groups (5.14 ± 0.53, 5.61 ± 0.21, and $5.92 \pm 0.38 \times 10^5/mm^2$, resp.) without significant difference. Ninety percent of all counted axons were myelinated (spared and remyelinating) in the healthy control group. This percentage dropped significantly to 34% after 4 weeks of cuprizone diet. Following 2 weeks of spontaneous remyelination, a significant ($P < 0.01$) increase in the percentage of myelinated axons was observed in the PBS (61%), eGFP-LV (60%), and IL-11-LV treated (65%) groups (Figure 5(d)). A close observation of the micrographs showed comparatively more structured tissue architecture in the IL-11-LV treated group (Figure 5(a)). Together these data confirm the potent remyelinating capability of IL-11 *in vivo*.

3.6. Therapeutic Overexpression of IL-11 Promotes Maturation of OLs during Remyelination. To reveal the underlying mechanism of this therapeutic effect, we investigated the effect of IL-11 on the maturation of OLs during 2 weeks of endogenous remyelination. Therapeutic overexpression of IL-11 increased the number of mature OLs in the corpus callosum. In the IL-11-LV treated group, the density of mature OLs in corpus callosum was significantly ($P < 0.05$) increased ($390.9 \pm 26.32/mm^2$) as compared to the PBS and eGFP-LV treated groups (275.8 ± 5.79 and $293.3 \pm 27.12/mm^2$, resp.) (Figures 4(b) and 4(d)). Moreover, the density of Iba-1^+Mac-2^+ microglia in the corpus callosum of IL-11-LV treated animals ($181.9 \pm 12.93/mm^2$) was significantly ($P < 0.05$) lower than that in the PBS and eGFP-LV treated groups ($304.9 \pm 20.20/mm^2$ and $278.0 \pm 23.40/mm^2$, resp.) during remyelination (Supplementary Figure 3).

4. Discussion

The appearance of inflammatory demyelinating lesions in the white matter of MS patients is thought to result from a coordinated autoimmune attack against CNS tissue. It is common knowledge that toxic mediators such as reactive oxygen species and proinflammatory cytokines produced by infiltrating immune cells and locally activated microglia are major contributors in the formation of these myelinating

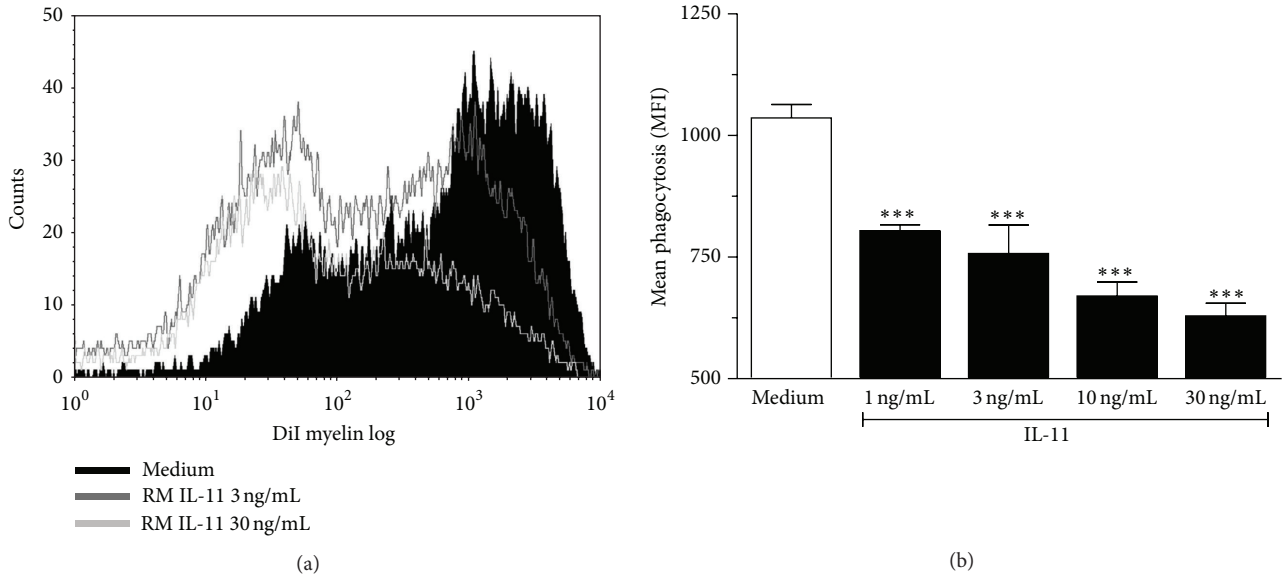

Figure 3: IL-11 inhibits myelin phagocytosis *in vitro*. BV2 cells were pretreated with increasing amounts of rmIL-11 for 12 h. Medium only was used as control. Subsequently cells were allowed to phagocytose DiI-labeled myelin for 90 min. (a) A representative single-parameter histogram showing IL-11-induced dose-dependent inhibition of DiI-labelled myelin phagocytosis. (b) Bar graph showing mean fluorescence intensity of DiI. The experiment was repeated three times and was performed in triplicate each time. Data is presented as mean ± SEM of triplicate determinations from three independent experiments. Statistical significance is analyzed by one-way ANOVA followed by Bonferroni's multiple comparison test. $^{***}P < 0.001$ as compared with medium only group.

lesions. It has become increasingly clear that both inflammatory cells and CNS resident cells are also able to produce factors that counteract the further spread of these lesions by promoting repair (for review see Kerschensteiner et al. 2009) [33].

Amongst others, different members of the gp130 family of cytokines are reported to be upregulated in MS lesions and likely represent an endogenous protective response mechanism to limit brain damage [13, 34]. LIF, OsM, and IL-11 are members of this family of cytokines that activate STAT3 through gp130 signaling. These cytokines exert effects on both immune cells and neural cells. Importantly, since they activate different receptors, LIF, OsM, and IL-11 can influence these parameters to different extents. During the past years, we investigated the effects of different gp130 family cytokines on distinct pathological processes in MS lesions. The experimental approach chosen is stereotactic application of lentiviral vectors to achieve a robust and stable expression and secretion within the CNS of adult mice, thereby mimicking the endogenous response in MS lesions. Moreover, this approach is able to discriminate systemic versus local effects of these neuropoietic cytokines on MS disease processes. Our study demonstrates that local overexpression of IL-11 is able to limit cuprizone-induced demyelination by reducing OLs cell death and decreasing microglial activation. Moreover, we demonstrated that IL-11 overexpression in established demyelinated brain regions enhances spontaneous remyelination.

In line with earlier observations [21, 31], a drop in the number of $CC1^+$ OLs was observed in the corpus callosum after 5 weeks of cuprizone diet, paralleled with a significant increase in the number of $NG2^+$ OPCs. Degeneration of OLs during cuprizone intoxication is thought to be the result of apoptosis-induced cell death [29, 31, 35]. To compensate for this OLs loss, OPCs are recruited to demyelinated areas and proliferate to replenish the OLs pool. Here we show that prophylactic overexpression of IL-11 limits mature OLs loss and prevents the consequent demyelination and proliferation of OPCs. Our *in vivo* findings are in line with previous reports demonstrating that IL-11 is promoting survival of OL in cultures through activation of STAT3 [13, 18]. Moreover, IL-11Rα knockout mice showed enhanced OL loss and demyelination in the lysolecithin-induced focal demyelination model [18] again underscoring the importance of IL-11R signaling in promoting survival of oligodendrocyte lineage cells. In addition to IL-11, LIF has been documented to promote survival of OL by inducing antiapoptotic 14-3-3 isoforms and activating Akt-signalling [36]. Together, these reports illustrate that different members of the gp130 cytokine family may have similar effects, although different pathways could be involved.

Demyelination in the cuprizone model also partially depends on the secretion of proinflammatory cytokines by microglia which are cytotoxic to OLs and neuronal cells [37, 38]. Indeed, Pasquini et al. showed that inhibition of microglial activation by minocycline significantly reduced cuprizone-induced demyelination [37]. Moreover, *in vivo* deactivation of microglia in EAE ameliorates neurodegeneration and demyelination [39]. Our finding that IL-11 expression reduced the activation of microglia *in vivo* could therefore also explain the reduction in OL loss and consequent demyelination. In line with that, IL-11 was previously reported

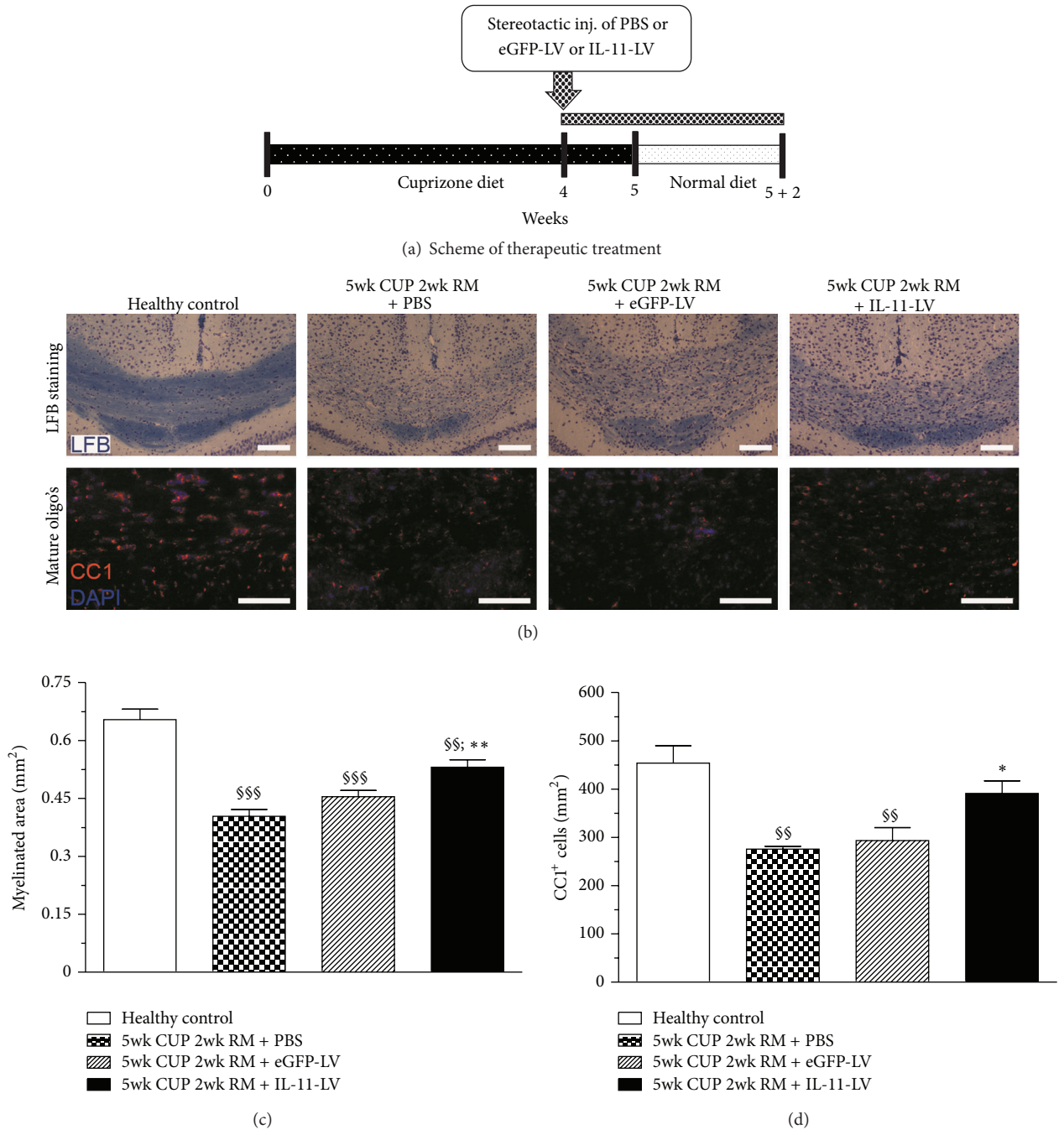

FIGURE 4: Scheme and the effect of *therapeutic overexpression of IL-11 on remyelination*. (a) Mice were fed with cuprizone diet for 4 weeks before PBS/eGFP-LV/IL-11-LV was administered stereotactically in the brain. Cuprizone diet was continued for more 1 week, before returning mice to standard chow for another 2 weeks. Panel (b) shows representative images of coronal brain sections from all groups, depicting myelinated area (LFB) and density of mature OLs (CC1). Quantitative analysis reveals that therapeutic overexpression of IL-11 (i) *enhances remyelination*, as illustrated by luxol fast blue staining depicting a larger myelinated area in the IL-11-LV treated group (c), and (ii) *facilitates the maturation of OL's* as revealed by a higher density of $CC1^+$ mature oligodendrocytes in the IL-11-LV treated group (d). Data is presented as mean ± SEM ($n = 5$ animals for the cuprizone treated groups; $n = 3$ for the healthy control group). Statistical significance was analyzed by one-way ANOVA followed by Bonferroni's multiple comparison test. $^{§§}P < 0.01$, $^{§§§}P < 0.001$ as compared to the healthy control group; $^{*}P < 0.05$, $^{**}P < 0.01$, as compared to the 5 wk CUP 2 wk RM + PBS. Quantification was performed in the corpus callosum of at least three coronal brain sections per mouse, chosen between anterioposterior coordinates from 0.3 to −1.5 mm in reference to the bregma. Scale bar: 200 μM.

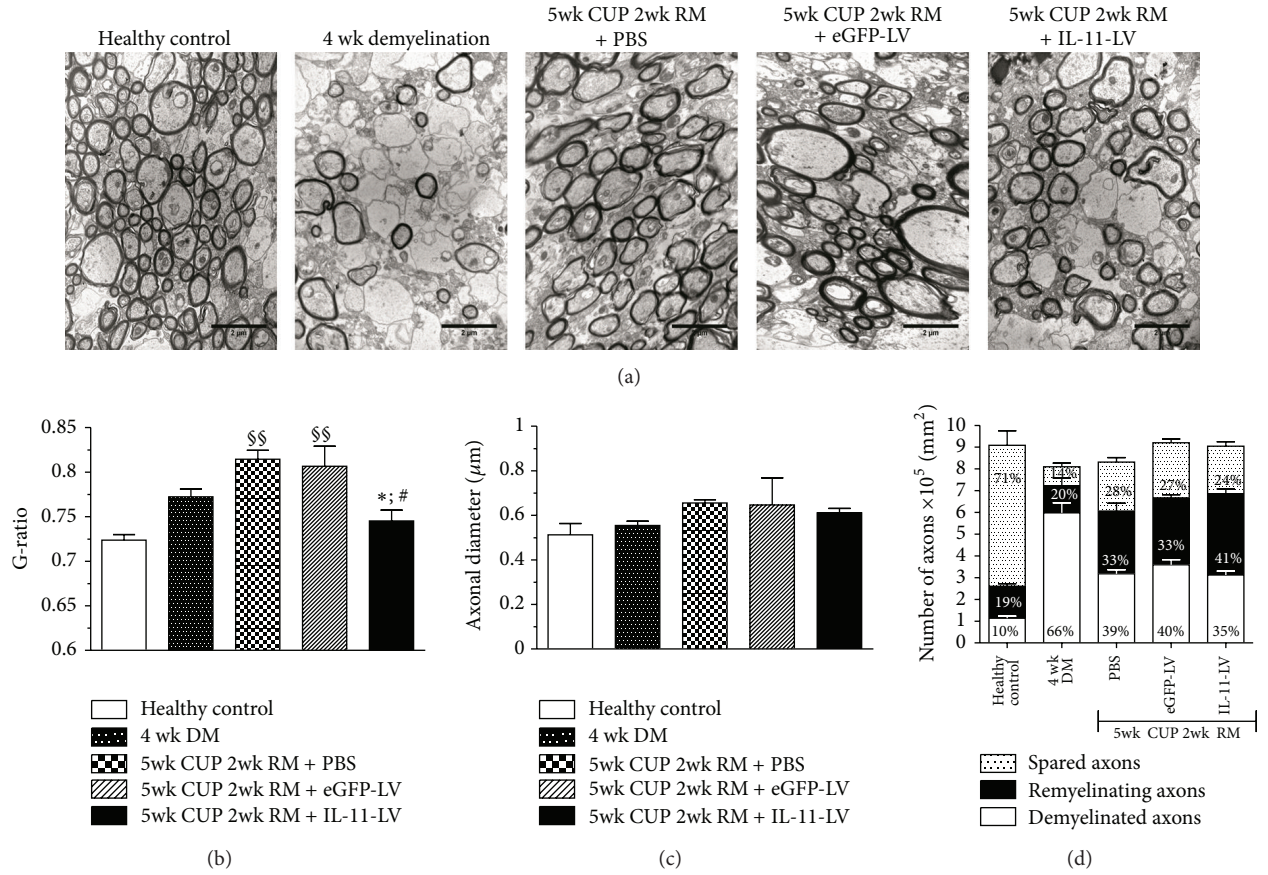

Figure 5: *Therapeutic overexpression of IL-11 promotes endogenous remyelination.* Representative images of electron micrographs (panel (a)) of midsagittal cross sections of the corpus callosum reveal significant demyelination after 4 weeks of cuprizone diet and remyelination after 5 weeks of cuprizone diet followed by 2 weeks of normal diet. Quantitative analysis shows that therapeutic overexpression of IL-11 (b) *enhances remyelination* as indicated by a decrease in G-ratio (c) without affecting the mean axonal diameter. (d) Quantitative and qualitative analysis reveals that 4 weeks of cuprizone treatment reduced the density of myelinated axons and again increased after the animals were allowed to remyelinate for two weeks. The values shown in the bars represent the percentage of axons of that category with respect to the total number of demyelinated and myelinated axons. Axons were categorized in different groups based on their individual G ratio: axons were defined as spared (G ratio < 0.74), remyelinating (0.74 < G ratio > 0.9), and demyelinated (G ratio > 0.90). Per mouse, quantification was performed in 5 randomly selected areas in the electron micrographs of midsagittal cross sections of the corpus callosum taken between anterioposterior coordinates from −0.3 to −1.5 mm in reference to bregma. Data is presented as mean ± SEM ($n = 3$ animals for each group). Statistical significance was analyzed by one-way ANOVA followed by Bonferroni's multiple comparison test. $^{\S\S}P < 0.01$, $^{\S\S\S}P < 0.001$ compared to healthy control group; $^{*}P < 0.05$ compared to 5 wk CUP 2 wk RM + PBS group; $^{\#}P < 0.05$ compared to 5 wk CUP 2 wk RM + eGFP-LV group; $^{@}P < 0.05$ compared to 4 wk DM group.

to inhibit tumor necrosis factor (TNF) α, IL-1β, IL-12, IL-6, and nitric oxide production by activated macrophages *in vitro* [17, 40]. Moreover, we demonstrated that IL-11 dose dependently diminished the uptake of myelin by the BV-2 microglial cell line *in vitro*. Since microglia are known to actively participate in stripping of myelin from axons, this could be a third mechanism by which IL-11 reduces demyelination. Further studies should determine in what way microglia participate in the observed IL-11-induced effects.

We further report that therapeutic overexpression of IL-11 resulted in an increased myelinated area (LFB) as compared to the control treatment groups after 2 weeks of endogenous remyelination. Furthermore, the mean G-ratio [32] of myelinated axons was significantly decreased in the IL-11 treatment group as compared to the control treatment groups. Since the mean axonal diameter was not significantly different among groups, the observed decreased G-ratio in the IL-11 treatment group reflects an increased thickness of myelin and thus enhanced remyelination. Since IL-11 increased the density of $CC1^{+}$ mature OLs *in vivo*, the increased remyelination could result from enhanced maturation of OPCs to myelinating OLs as shown by Zhang et al. *in vitro*. Earlier *in vitro* studies already reported promyelinating effects of IL-11. Indeed, IL-11 increased the numbers and promoted the maturation of myelinating cells in CNS cocultures [13, 18].

5. Conclusion

Our report shows that IL-11 is of therapeutic interest for diseases with a demyelinating component. Moreover, due to

its potent anti-inflammatory effects on different immune subsets (T cells, DC, and macrophages) [15, 18, 19], IL-11 seems to be an ideal tool to intervene in MS disease processes at different levels. This hypothesis is supported by the group of Gurfein et al. that reported a significant increase in EAE disease severity and neuropathology in IL-11Rα knockout mice [19]. They further demonstrated that systemic IL-11 treatment was shown to regulate EAE disease via a combination of immunoregulation and a prosurvival effect on oligodendrocytes. In contrast, IL-11 signalling was recently found to be dispensable in spinal cord injury (SCI) [41], indicating that IL-11 may not be protective in all disease settings.

Conflict of Interests

The authors certify that there is no conflict of interests for any of the coauthors with any financial organization regarding the material discussed in the paper.

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Clinical Study

Growth Arrest Specific Gene 6 Protein Concentration in Cerebrospinal Fluid Correlates with Relapse Severity in Multiple Sclerosis

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Background. Growth arrest specific gene 6 (Gas6) protein enhances survival of oligodendrocytes and neurons, and it is involved in autoimmunity. Therefore, we aimed to verify whether cerebrospinal-fluid (CSF) Gas6 concentration may represent a biomarker of disease activity in multiple sclerosis. *Methods*. Sixty-five patients who underwent a spinal tap during relapse of relapsing/remitting multiple sclerosis (RR-MS)(McDonald-criteria) were studied. Forty patients affected by noninflammatory/nonautoimmune neurological diseases served as controls. Relapse was defined according to Schumacher criteria. Symptoms were grouped according to Kurtzke-Functional System (FS). Clinical characteristics of the relapse, duration, Expanded-Disability-Status Scale (EDSS) change, number of FS involved, completeness of recovery, age, steroid therapy, were categorised. Patients were followed at 6-month intervals to assess relapse rate and EDSS progression. Gas6 was measured (CSF, plasma) by in-house-enzyme-linked immunoassay (ELISA). *Results*. Higher CSF Gas6 concentrations were observed in relapses lasting ≤60 days (8.7 ± 3.9 ng/mL) versus >60 days (6.5 ± 2.6) or controls (6.5 ± 2.4; $P = 0.05$), with ≤2 FS involved (8.5 ± 3.8) versus >2 FS (5.6 ± 2.5) ($P < 0.05$) and EDSS change ≤2.5 points (8.8 ± 3.7) versus >2.5 (6.5 ± 3.5) ($P = 0.04$). Conversely, CSF Gas6 was not predictive of the completeness of recovery. Plasma and CSF concentrations were not related ($R^2 = 0.0003$), and neither were predictive of relapse rate or EDSS progression after first relapse. *Conclusions*. CSF concentration of Gas6 is inversely correlated with the severity of relapse in RR-MS patients but does not predict the subsequent course of the disease.

1. Introduction

Multiple sclerosis (MS) is an immune-mediated disorder of the central nervous system (CNS) determined by an inflammatory aggression to the myelin of neuronal fibers. The pathogenesis of MS involves complex interactions between many cell types, including both the adaptive and innate immune systems. In this context, activated macrophages and microglial cells mediate myelin degradation and oligodendrocyte damage by producing proinflammatory cytokines such as tumor necrosis factor- (TNF-) alpha and interferon-gamma [1]. For this reason, molecules involved in dampening of macrophages activation could be involved in MS pathogenesis.

Gas6 (growth arrest specific 6), a multimodular secreted protein, binds and activates receptors belonging to the Tyro-3 tyrosine kinase receptor family (Tyro-3, Axl, and Mer) also known as TAM receptors [2–5]. Gas6/TAM interaction is involved in several physiological processes, including cell migration, adhesion, cell growth, and survival [6]. Recently, it has been claimed that Gas6/TAM system may be involved in the pathogenesis of autoimmune diseases [7]. This hypothesis is based on the observation that knock-out mice for TAM receptors display aberrant proliferation of T and B

lymphocytes, with diffuse tissue infiltration and autoimmune manifestations including vasculitis, lupus-like lesions, and autoantibodies. This phenotype is likely the consequence of monocytes-macrophages hyperactivity [8]. The importance of Gas6 in controlling the immune response is supported by the finding that Gas6 inhibits macrophage activation and cytokine production, reducing interleukin (IL)-6 and TNF-alpha secretion through the activation of Mer receptors [9].

Direct evidences link Gas6 to autoimmune processes within the central nervous system (CNS). Gas6 and its TAM receptors are expressed in the brain of embryonic and adult rats proportionally to synaptogenesis of cerebral tissues [10–12]. In addition, Gas6 promotes proliferative and antiapoptotic effects on several CNS cell types as hippocampal and cortical neurons [11, 13], Schwann cells [14] and, in particular, oligodendrocytes, which are protected from apoptosis trough Axl/PI3-K/Akt activation [15, 16]. Binder and colleagues have also shown that the absence of Gas6 affects the efficiency of remyelination following a demyelinating insult, induced by cuprizone and that defect of remyelination is accompanied to reduction in the number of mature oligodendrocytes [17]; moreover the administration of recombinant human Gas6 to the corpus callosum enhanced the myelin repair in the same mouse model [18]. These observations suggest that the Gas6/TAM system may be involved both in the regulation of the survival of neuronal and glial cells, in particular of those involved in myelination, and the control of the innate immune response, two paramount processes in MS pathogenesis. Based on these premises, in the present study we measured CSF and plasma Gas6 protein during a relapse of multiple sclerosis in relation to relapse clinical features and severity scores as Kurtzke-Functional-System (FS), to verify their usefulness as a biomarker of disease course.

2. Methods

2.1. Patients. Sixty-five consecutive patients admitted to an academic referral centre from 2001 to 2005 with a diagnosis of clinically isolated syndrome (CIS) or Relapsing-Remitting (RR) MS were studied. CIS was defined as the occurrence of an acute or subacute event of the CNS affecting the optic nerves, brainstem, or spinal cord of presumed inflammatory demyelinating origin in a patient with no history suggestive of an earlier demyelinating episode [19]. McDonald criteria [20] were used to diagnose MS. Inclusion criteria were age ≥18 years, first visit to the MS Centre, diagnostic lumbar puncture during the relapse, no treatment with corticosteroids prior to the lumbar puncture, and no prior treatment with any immunomodulant drug. Patients with primary progressive MS were excluded. The majority of patients ($n = 45$) were sampled during their first relapse; the others were observed and sampled at their second ($n = 16$) or third relapse ($n = 4$); moreover at sampling 22 were CIS and 43 RR-MS. After the first visit, 55 patients (10 patients were lost to follow-up) were prospectively followed and visited thereafter every 6 months; the follow-up ended on July 31, 2009 (Table 1). Follow-up duration ranged from 2.5 to 11 years (mean 5.9 years; SD 1.8). At the end of this follow-up period, the diagnosis was CIS in 9 cases and RR-MS in 42, while 4 patients had already entered secondary progression.

Table 1: Features of the population studied.

Population studied	Number of patients
First relapse/second/third relapse	45/16/4
CIS/RR-MS	22/43
Patients with follow-up ≥2.5 years	55

CIS: clinically isolated syndrome; RR-MS: relapsing remitting multiple sclerosis.

The study was approved by a local Ethical Committee and conducted in strict accordance with principles of the Declaration of Helsinki. Each patient gave a written informed consent before the lumbar puncture to the use of his/her CSF for experimental evaluation and to maintain data in the database.

2.2. Relapse Definition and Evaluation. Relapse was defined as "acute or subacute occurrence, recurrence or worsening of symptoms of neurologic dysfunction attributable to MS, lasting more than 24 hours after a period of at least 30 days of improvement or stability" [21]. Neurological deterioration of preexisting symptoms accompanied by fever was not considered a relapse. Paroxysmal episodes were considered as relapse when multiple and occurring over not less than 24 hours. Symptoms occurring within a month after the initial symptoms of relapse were considered to be part of the same episode. Patients data were collected at the first visit at the centre during the relapse and also at 3, 7, and 30 days after the relapse onset. For each relapse, type of symptoms, signs, and dates of occurrence were collected, as previously described [22]. Briefly, symptoms and signs were grouped to fit in with the Kurtzke Functional System (FS) [23]. The score for each FS and the total EDSS score were calculated at onset, at maximum worsening (zenith) and at the first examination after the day of maximum improvement of the relapse. We correlated the following factors with Gas6 concentration: relapse duration and severity, recovery, gender, age at the onset of the relapse, number of affected FSs, Link index (≤0.70/>0.70), annualized relapse rate, and annualized EDSS progression. The relapse duration was calculated as the time between the date of onset of the first symptom and the date of maximum improvement of the last symptom. The severity of the relapse was calculated as the difference between the EDSS score at the day of maximum worsening and the EDSS score before the onset of the relapse; the lowest ΔEDSS was 0.5 (1.0 for the first relapse). Recovery was evaluated at the date of maximum improvement and classified as complete (EDSS = 0) or incomplete (EDSS ≥ 1). Annualized relapse rate was calculated as the number of relapses during the follow-up divided by the months of the follow-up. Annualized EDSS progression was calculated as the difference between the EDSS score of the last follow-up visit available and the EDSS score after the first relapse, divided by the years of follow-up.

2.3. CSF and Plasma Sampling and Gas6 Measurement. CSF samples of MS patients were drawn within 90 days from

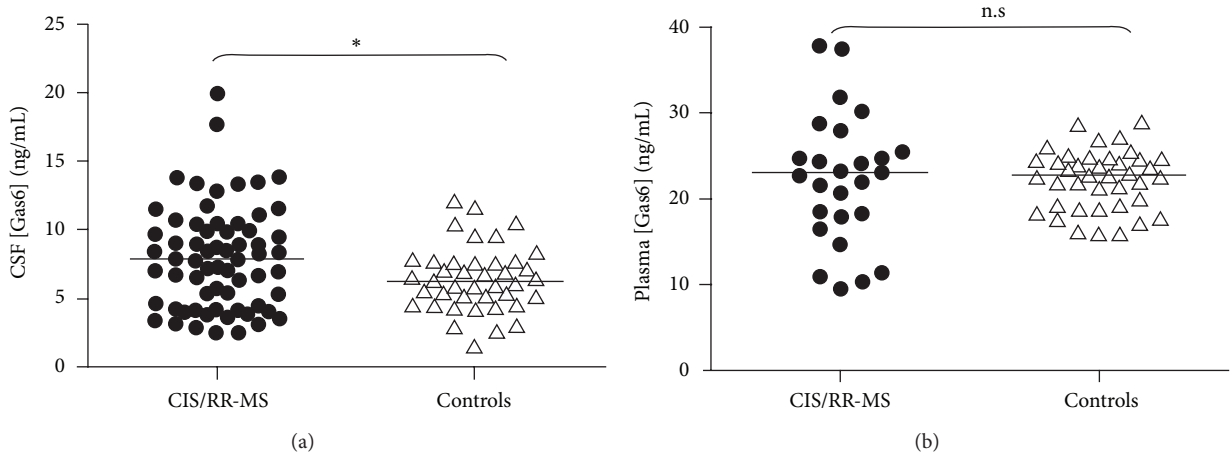

Figure 1: Gas6 concentration in CSF of patients with MS. (a) The scatter plot displays CSF Gas6 concentrations of patients with MS (black circles) with respect to controls (white triangles). * indicates $P < 0.05$ in statistical comparison of the means (Student's t test); the horizontal bar indicated the mean value. (b) The scatter plot displays plasma Gas6 concentrations of patients with MS (black circles) with respect to controls (white triangles). The horizontal bar indicated the mean value.

the symptoms onset and before complete resolution of the relapse; 29 patients underwent also a blood draw in the same day of the spinal puncture. CSF and plasma samples were obtained from a biobank including patients affected by a noninflammatory/nonautoimmune neurological diseases (NINAD) such as ischemic stroke, amyotrophic lateral sclerosis, headache, and psychiatric conditions simulating neurological diseases or otologic dizziness. CSF and plasma samples of cases and controls were stored at −30°C. Gas6 was measured by a sandwich ELISA developed and validated in our laboratory for human plasma samples [24, 25] and with the same method modified for CSF assay as previously described [26]. Samples from MS patients had been collected between 2001 and 2005, appropriately aliquoted and stored at −80°C to be tested for Gas6 concentration between 2009 and 2010. Samples from NINAD controls were also collected along the years from 2001 and 2005, processed and stored similarly, and used for Gas6 testing in 2007; these latter data had already been published [26]. Briefly a 96-well plate (NUNC ImmunoPlates MaxiSorp F96, NUNC, Hereford, UK) is coated overnight with anti-human-Gas6 primary antibody (goat polyclonal affinity purified IgG, R&D Systems, Minneapolis, MN. USA). The antigen is detected by a secondary biotin conjugated antibody (Biotinylated anti-human Gas6 antibody, R&D Systems, Minneapolis, MN. USA), a streptavidin-peroxidase conjugate (Sigma, St. Louis, MO, USA), and TMB (3,3′,5,5′-tetramethylbenzidine, Sigma, St. Louis, MO, USA). The reaction is blocked with sulphuric acid 1.8 and absorbance detected at 450 nm with a reference wavelength set at 570 nm. Optical density is fitted versus nominal concentration by applying a four-parameter logistic regression to the calibration curve prepared in Gas6 depleted plasma or in BSA (Bovine serum albumin, further purified fraction V, ≥98%, Sigma, St. Louis, MO, USA). The method has been validated according to the Food and Drug Administration guidelines for inter- and intra-assay % coefficient of variation (%CV) for Gas6 measurement in human plasma and CSF (all %CVs were within 15% with negligible matrix effect). There was no cross-reactivity with human protein S (MP Biomedicals, Solon, OH, USA). The lowest quantification limit was 0.26 ng/mL [24–26].

2.4. Statistical Analysis. Data were collected and stored in an electronic database to be analyzed with the Statistica statistical software program, release 10 (StatSoft, Tulsa, OK, United States). The Shapiro-Wilk test was performed to assess normality for any continuous variable analyzed. The measures of central tendency and dispersion used throughout the paper were means and standard deviations for continuous variables with normal distribution. Accordingly, normally distributed variables were compared among groups by means of the Student's t-test, ANOVA, and post hoc comparisons of the means (Tukey's post hoc test for nonhomogeneous samples/test of Spjotvoll-Stoline). Categorical variables were analyzed with Pearson chi-square test and stepwise logistic regression to identify independent predictive factors of CSF Gas6 variations. A level of 0.05 (two-sided) was chosen to indicate statistical significance.

3. Results

Mean CSF Gas6 concentration was nearly equal in CIS with respect to RR-MS patients (7.8 ± 3.2 ng/mL versus 7.9 ± 3.8, Student's t-test, P = n.s.) but significantly higher in CIS/RR-MS (n = 65; 37 females) patients with respect to controls (n = 40; 22 females) (7.9 ± 3.7 ng/mL versus 6.5 ± 2.4 ng/mL resp., $P < 0.03$) while plasma Gas6 concentration was not significantly different between groups (Student's t test, P = n.s.) (Figure 1).

Figure 2 shows mean CSF Gas6 concentration in relationship to the duration of relapse. CSF Gas6 concentration was significantly higher in shorter relapses (relapses lasting ≤60 days 8.7 ± 3.9 ng/mL, versus >60 days

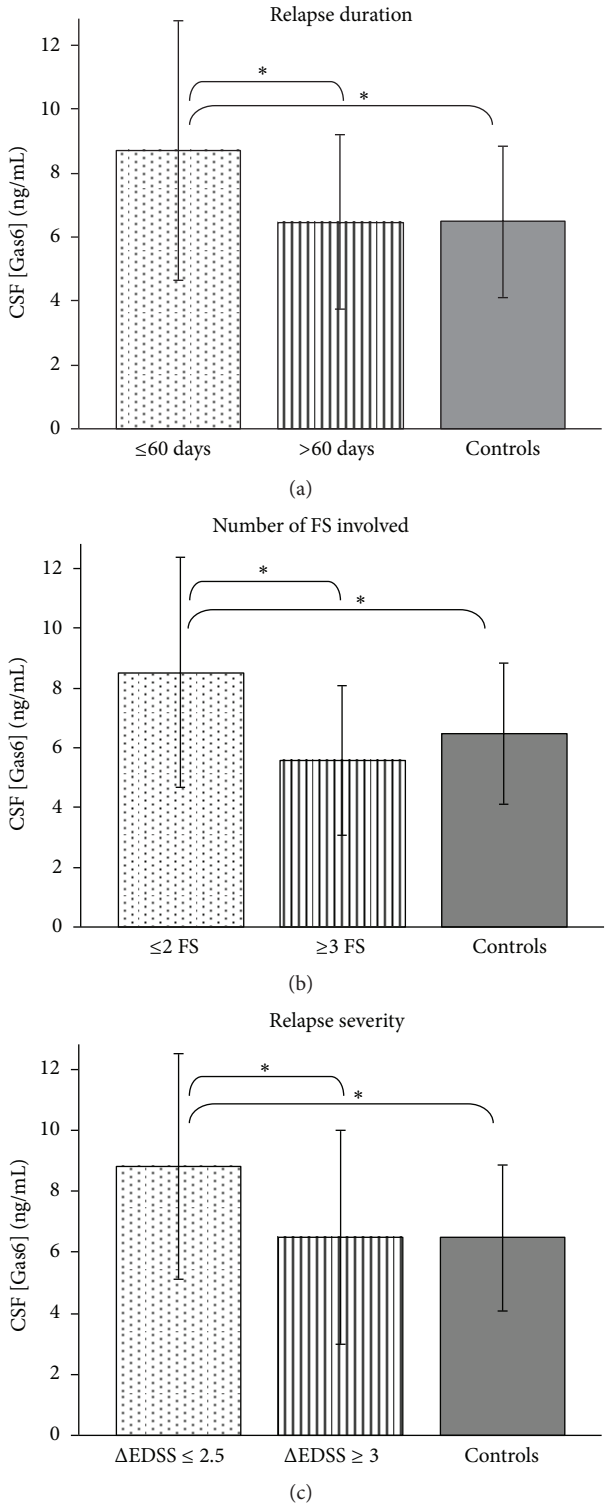

Figure 2: Gas6 concentration in CSF of patients with MS in relation to relapse features. The graph shows means and standard deviations of CSF Gas6 concentrations in relation to relapse duration: ≤60 days (dots), >60 days (vertical lines) and controls (gray) (panel a); number of FS involved during the relapse: ≤2 (dots), ≥3 (vertical lines) and controls (gray) (panel b); and EDSS score variation at maximum worsening (severity): ≤2.5 (dots), ≥3 (vertical lines) and controls (gray) (panel c). *indicates $P \leq 0.05$ in statistical comparison of the means (Tukey's post hoc test).

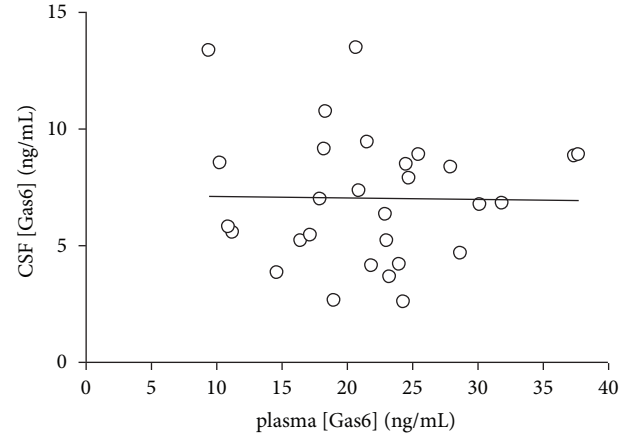

Figure 3: Dissociation between CSF and plasma Gas6 concentration. The figure displays the absence of correlation between CSF and plasma Gas6 concentration measured simultaneously during the first relapse in MS patients. The line indicates the regression equation: CSF Gas6 = 0.007 · plasma Gas6 + 7.1, $r^2 = 0.0003$, P = ns.

6.5 ± 2.6 ng/mL and controls 6.5 ± 2.4 ng/mL; ANOVA: $F = 5.8$, $P < 0.004$; Tukey post hoc test, $P = 0.05$), in relapses with fewer FS involved (≤2 FS 8.5 ± 3.8 ng/mL versus ≥3 FS 5.6 ± 2.5 ng/mL versus controls; ANOVA: $F = 6.7$ $P < 0.002$; Tukey post hoc test, $P < 0.05$) and in relapses with lower severity (ΔEDSS ≤ 2.5 8.8 ± 3.7 ng/mL versus ≥3 6.5 ± 3.5 ng/mL versus controls; ANOVA: $F = 6.6$ $P < 0.002$; Tukey post hoc test, $P < 0,04$). In particular, CSF Gas6 concentration was inversely proportional to the EDSS score of the relapse ($R^2 = 0.11$, $P < 0.01$) especially in pyramidal or cerebellar ones ($R^2 = 0,25$, $P < 0,003$). On the other hand, CSF Gas6 concentration did not change according to completeness of recovery, being similar in patients with or without complete recovery (8.2 ± 4.0 ng/mL versus 7.6 ± 3.5 versus controls, ANOVA, P = n.s). Alternatively, CSF Gas6 did not vary according to age, therapy, Link index (P = n.s.). Conversely, plasma Gas6 concentration was not related to any of the clinical variables listed above (ANOVA, P = n.s). Indeed, there was no relation between CSF and plasma Gas6 concentration ($R^2 = 0.0003$, P = n.s), as shown in Figure 3. In stepwise logistic regression, relapse duration and number of FS involved were significantly associated with Gas6 concentration (F : 13.3 and 6.5 resp., $P < 0.02$). Treatment allocation according to relapse features is shown in Table 2.

Follow-up data for a minimum of 2.5 years were available for 55 out of 65 patients and are presented in Table 3. Gas6 concentration did not differ according to annualized relapse rate either in CSF (Student's t test: ≥0.25/year 8.4 ± 4.6 versus <0.25/year 8.0 ± 3.2 ng/mL, P = n.s.) or in plasma (21.5 ± 6.4 versus 21.3 ± 12.4, P = n.s) and according to annualized EDSS progression either in CSF (≥0.2 EDSS points/year 7.9 ± 3.1 versus <0.2 8.1 ± 4.8, P = n.s.) or in plasma (21.8 ± 8.8 versus 23.4 ± 7.6 ng/mL, P = n.s). Moreover, neither CSF nor plasma Gas6 concentration correlated to annualized relapse

Table 2: Treatment allocation according to relapse features.

Relapse features	Type of treatment			χ^2 analysis
	No	Oral PDN*	IVMP**	
Duration ≤60/>60 days	22/10	7/5	16/5	1.15, P = n.s.
Number of FS involved (≤2/>2)	26/7	8/4	16/4	0.88, P = n.s.
Severity, ΔEDSS (≤2.5/>2.5)	22/11	7/5	11/9	0.78, P = n.s.

PDN: prednisone, IVMP: intravenous methylprednisone, FS: functional system, and ΔEDSS: variation from baseline of Expanded-Disability-Status Scale.
*Oral prednisone was administered at 50 mg/day for the first 5 days then 25 mg/day for the following 5 days and 12.5 mg/day for further 5 days and then discontinued.
**Intra venous methylprednisone was administered at 1000 mg/day i.v. infusion for 5 consecutive days.

Table 3: Follow-up cohort features.

	M ± SD	Median
Follow-up duration (years)	5.9 ± 1.8	5.2
Annualized relapse rate (relapse/year)	0.32 ± 0.25	0.25
Annualized relapse progression (ΔEDSS/year)	1.6 ± 1.0	1.0

rate or EDSS progression in linear regression analysis (data not shown, P = n.s.).

4. Discussion

The present paper shows that CSF Gas6 concentration is slightly higher in patients suffering a first relapse of MS than in control patients. Interestingly, a significant and substantial increase in CSF was observed in patients suffering from shorter, less severe, and less FS involved relapses. Conversely, patients who suffered from more severe relapse did not show any variations of CSF Gas6 concentration with respect to control subjects. This apparent paradox is reconciliated by considering Gas6 as a protective protein, able to limit inflammatory demyelination. This hypothesis is sustained by several in vitro and in vivo studies that indicate for Gas6 a role as inhibitor of macrophages activation [7, 9] and as a survival factor for neurons and oligodendrocytes [11, 15, 16]. Indeed, Gas6 knock-out mice challenged with cuprizone display increased demyelination damage with greater lesions and a higher number of apoptotic oligodendrocytes, infiltrating macrophages and microglial cells [6]. In absence of Gas6 there is a delay in remyelination of the lesions induced by cuprizone [17]. Moreover, Gas6 concentration is raised in the CSF of patients suffering from CIDP, a chronic autoimmune demyelinating disease of the peripheral nerves which shares some similarities with MS [26]. In summary, our results fit well the data in the literature, suggesting that MS patients with higher CSF Gas6 might be able to limit the extent of inflammatory demyelination. On the other hand Gas6 elevation in CSF seems to be related to relapse features rather than to the presence of MS itself. Additionally, since an altered Gas6/TAM ratio has been evidenced in established MS lesions [27], an impaired expression of TAM-receptor in absence of alteration of Gas6 concentration may concurr to influence the disease course. Further studies are needed to address this hypothesis.

The mechanisms by which Gas6 might exert this putative protection by the effects of inflammatory demyelination remain speculative. Gas6 could act on macrophages and/or glial cells; this hypothesis is supported by the observations indicating that Gas6 downregulates the production of proinflammatory cytokines, such as IL-6 and TNF-alpha, and enhances the phagocytosis of cellular debris and apoptotic bodies through Mer receptor by activated macrophages, dendritic and microglial cells [7, 9, 28–30]. Thus, Gas6 may be able to limit the activation of the innate immune response and, consequently, the aggression to myelin fibers favoring cell debris and apoptotic bodies clearance which are, in turn, inflammatory triggers. Since mononuclear activation with cytokine production is crucial in the amplification of the autoimmune aggression to myelin and the failure to clear apoptotic cells is widely considered a trigger of autoimmunity, Gas6 may have an important role in this context. Alternatively, Gas6/TAM interaction could be relevant in processes of recovery after inflammatory demyelination, since it is well demonstrated that it enhances the survival of oligodendrocytes and neurons [11, 15], as happening for other cell lines [31], and mice lacking Gas6 have demyelinating lesions with a higher proportion of apoptotic oligodendrocytes during cuprizone-induced demyelination [6]. Consequently, Gas6 may participate actively in oligodendrocyte survival after myelin damage favoring a prompt repair. It is conceivable that patients who suffer from more severe relapse and who do not present an increase in CSF Gas6 concentration may have a defect in Gas6 expression and, consequently, present a defect in mechanisms of self-limitation to immune aggression to CNS or in myelin damage repair. It must be pointed out, however, that we were unable to identify a relation between Gas6 concentration in CSF and lack of complete recovery after the relapse, which could be considered the clinical correlate of an efficient recovery system.

The putative role of Gas6 in the control of immune aggression in the nervous system is strengthened by the finding that plasma and CSF Gas6 concentration are unrelated in MS patients; therefore, the increase of Gas6 in the CSF was not dependent on an increased plasma concentration and/or the breakdown of the blood-brain barrier, but likely on production by inflammatory cells within the CNS. These results are in line with previous results from our group concerning autoimmune polyneuropathies [26, 32]. Moreover, the relation between CSF Gas6 and EDSS score was stricter in pyramidal and cerebellar relapses, possibly because EDSS

is more sensitive to the damage extension in pyramidal and cerebellar relapses with respect to other symptoms that are mainly subjective and less easy to score.

Although the sample size was small and only a few patients had an active disease with a significant number of relapses or severe disability progression, our data suggest that measuring Gas6 concentration in CSF of MS patients can only be useful to predict the evolution of a first relapse but not the subsequent disease course. Thus, Gas6 may be important in limiting the acute inflammatory demyelination during a relapse, but less than so in the pathogenic mechanisms that determine relapse recurrences and cumulative disability.

In conclusion, CSF Gas6 protein concentration is significantly increased in milder MS relapses, suggesting an involvement of this protein in limiting autoimmune demyelinating processes or in favouring myelin repair after damage. Gas6 measurement in CSF during a relapse can be useful to predict how a relapse will evolve, but not the disease course in the long term. Further studies are warranted to clarify the Gas6 and TAM receptors role in the pathogenesis of MS-relapses and its clinical applications.

Conflict of Interests

The authors declare no conflict of interest.

Acknowledgments

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Review Article

Role of Scavenger Receptors in Glia-Mediated Neuroinflammatory Response Associated with Alzheimer's Disease

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It is widely accepted that cells serving immune functions in the brain, namely, microglia and astrocytes, are important mediators of pathological phenomena observed in Alzheimer's disease. However, it is unknown how these cells initiate the response that results in cognitive impairment and neuronal degeneration. Here, we review the participation of the immune response mediated by glial cells in Alzheimer's disease and the role played by scavenger receptors in the development of this pathology, focusing on the relevance of class A scavenger receptor (SR-A) for Aβ clearance and inflammatory activation of glial cell, and as a potential target for Alzheimer's disease therapy.

1. Introduction

Alzheimer's disease (AD) is the most prevalent form of dementia, being usually diagnosed in people over 65 years, although the less prevalent early-onset forms of AD develop at earlier ages [1]. AD shows an increasing prevalence mainly due to the population aging. Dementia prevalence rises exponentially, doubling the rate of AD every 5 years after the age of 60 with a 15-fold prevalence increment from-age-60 to 85 years [2].

The pathophysiology of this dementia is characterized by the extracellular accumulation of amyloid-β (Aβ) and the intracellular formation of neurofibrillary tangles of the Tau protein in neurons, in association with neuronal dysfunction and cell death in some brain areas as the hippocampus. Although Aβ has been considered to be the main agent implicated in AD pathogenesis, it is still uncertain if Aβ plaques are causative for AD or a consequence of its pathological changes.

There are several hypotheses that attempt to explain the origin of AD [3], although the most popular is still the "Aβ cascade hypothesis" [4], which considers Aβ as the key pathogenic factor. The "inflammation hypothesis" [5] and the "glial dysfunction hypothesis" [6] have lately gained increased support. With some differences, both of them state that Aβ accumulation is a consequence of the dysregulated activation of glial cells, which in turn induce an inflammatory response, alter their Aβ-clearance activity, and mediate the neurotoxic effects of Aβ [7, 8].

2. Glial Dysfunction Hypothesis

It is widely known that aging, the most robust risk factor for AD, is also strongly associated with a progressive increment on the inflammatory state of the organism. Inflammation induces a large amount of cell changes at multiple levels, including microglial cells [9], and, as it will be discussed in the next section, microglial cells also become more neurotoxic in response to inflammatory states [10]. Whereas the inflammation hypothesis considers that hyperreactive microglia is the major contributor to the adverse events associated with AD, the glial dysfunction hypothesis suggests that impairment of normal glial functions, meaning qualitative changes, and not only quantitative changes on microglial cell

activation, are responsible for the synaptic dysfunction and the neurodegenerative process observed in AD [6].

As it will be further discussed, glia are the scavenger cells of the brain. By having a reduced capability to clear Aβ [11], Aβ accumulates and microglial cells become activated and create a cytotoxic environment that induce a vicious circle that potentiates a neuroinflammatory state and neurotoxicity [12, 13]. Because the impairment of Aβ clearance induce the accumulation of the peptide even if there are no changes in Aβ production, this hypothesis states that Aβ accumulation would be a consequence and not a cause of the pathogenic changes leading to AD [14, 15].

3. Neuroinflammatory Response in AD

3.1. Microglial Cell Response. There is robust evidence showing high levels of inflammatory mediators in the brain of AD patients. Around senile plaques, a strong presence of TNF-α, IL-1β, IL-6, monocyte chemoattractant protein-1 (MCP-1), complement proteins as C1q, C1r, C2, C3, C4, C5, C6, C7, C8, and C9, C-reactive protein, and class II major histocompatibility complex antigen HLA-DR is observed [16–20]. When the effect of inflammatory cytokines over the production of Aβ was evaluated, it was demonstrated that exposure to cytokines such as IL-6 and IL-1, increase neuronal amyloid precursor protein (APP) mRNA expression [21]. In addition, glial cultures obtained from rapid brain autopsies of AD patients stimulated with Aβ show an increased release of prointerleukin-1β (pro-IL-1β), IL-6, IL-8, TNF-β, MCP-1, macrophage inflammatory peptide-1α (MIP-1α), and macrophage colony-stimulating factor (M-CSF) [22], corroborating the inflammatory activation of glial cells as part of the physiopathology of AD. Despite of this, *in vitro* studies have demonstrated that Aβ potentiates inflammatory activation of microglia [23], with different forms of Aβ showing distinct patterns of cytokine release; for instance, soluble forms of $A\beta_{1-40}$ stimulate specifically the release of IL-1β, while $A\beta_{1-42}$ induces the release of IL-1α and IFN-γ [24].

Also, immunohistochemical studies of the brain of AD patients have shown the presence of reactive microglia closely associated with senile plaques [20, 25]. The exposure of microglia to a soluble form of APP (sAPP) induces an increase of activation markers in microglia and enhances their production of neurotoxins [26]. More specifically, Aβ stimulates the NFκB-dependent pathway [27], which in turn induces the cytokines production by microglia and initiates the neurotoxic effects mediated by these cells [17].

Using mouse models of AD, Simard et al. showed that amyloid plaques are capable of chemoattracting immune cells from the bone marrow into the brain parenchyma, which adopt a microglial cell phenotype in the brain and suggested that these immigrant cells are the main responsible for Aβ plaque clearance [28]. Microglial cell-associated Aβ clearance was originally shown by incubating murine microglia with fluorescent-labeled Aβ [11]. The study also established the participation of scavenger receptors (SRs) in this process by demonstrating that coincubation with an excess of SRs ligands blocked the phagocytosis of Aβ. Also, the use of Chinese hamster ovary (CHO) cells transfected with scavenger receptors of class A (SR-A) and class B (SR-B) significantly potentiated the uptake of Aβ [11], situating SRs as the principal receptors responsible for senile plaques clearance.

Although the mechanistic factor involved in the association between AD and aging is still an unsolved question, there is evidence pointing out to microglial aging as well as other age-related changes as responsible for this correlation [9]. Studies of adult cortical cells have shown a reduced capacity of aged microglia to phagocytose Aβ [29]. This decrease in phagocytic activity was mainly favored by a proinflammatory state [30]. In addition, it has been shown that Aβ has cytotoxic effects only in aged individuals, with no Aβ-induced neurodegeneration observed in the brain of young animals [31].

All these microglial-mediated effects observed in AD are contrasted to the normally neuroprotective role of microglia, which involves the phagocytic capacity responsible for brain surveillance from infection and physical injuries, the supportive role implicated in neuronal survival by secreting nerve growth factors, and the contribution in creating a microenvironment for central nervous system (CNS) regeneration [32, 33]. These changes in normal microglial cell functions are usually explained as a switch in the inflammatory state of the cell: during aging (and possibly also in AD), microglia change from an M2 activation state, characterized as an anti-inflammatory phenotype associated with wound healing, to an M1 state, which is a proinflammatory activation state related to the production of inflammatory cytokines, chemokines, and reactive intermediates [34].

This evidence leads us to propose that aging, which is commonly associated with a progressive inflammatory state of the brain, can be one of the most important causes of the defective clearance of neurotoxic Aβ, which in turn favors the overstimulation of the immune response, creating a positive feedback that leads to neuronal dysfunction, neurodegeneration, and the progressive development of neurodegenerative diseases like AD.

3.2. Astrocytes Response. As for microglia, astrocytes have also been observed in close proximity to senile plaques of AD patient brains [35]. Moreover, Aβ is capable of stimulating the production of MCP-1 in astrocytes [36], having an important role in chemotaxis for attracting immune cells to the senile plaque.

In AD patient brains, an upregulation of IFN-γ receptor (IFNGR) on activated astrocytes has been observed, where treatment with INF-γ resulted in reduced cell viability [37], suggesting that activated astrocytes can become neurotoxic at least under certain conditions of inflammatory stimulation.

Under physiological conditions, astrocytes are the cells that maintain the brain integrity: they provide metabolic support for neurons; are capable of sensing and modulate the neuronal environment; regulate the synaptic levels of glutamate, ion concentrations, and the acid-base balance; synthesize and release antioxidant molecules; participate in

the formation of the blood brain barrier; and function as immune competent cells by acting in the clearance of cell debris and as antigen presenting cells [38]. However, the effects mediated by astrocytes in AD are mainly harmful [39], which reveals the dual properties of astrocytes depending on the cellular context in which they are immersed.

In AD animal models, it has been demonstrated that astrocytes surrounding Aβ plaques are immunopositive for IL-6 [40]. In the same line of evidence, *in vitro* experiments show that Aβ exposure has differential immune effects in astrocytes depending on the peptide conformation: oligomeric Aβ induce transient high levels of IL-1β with a fast decrease, increasing nitric oxide (NO) production, inducible nitric oxide synthase (iNOS) and TNF-α expression, consistent with an early inflammatory response, while fibrillar Aβ induces persistent increased levels of IL-1β that remains over time, corresponding to a more chronic response [41]. The release of IL-1β by Aβ-stimulated astrocytes promotes the release of IL-1β, IL-6, and TNF-α by microvascular endothelial cells, suggesting that astrocytes-cytokine release also plays a role in neuroinflammation and endothelial response that contribute to AD progression [42].

In vitro studies have also shown that astrocytes exposed to Aβ present sporadic cytoplasmic calcium signals that correlate with the death of adjacent neurons, an effect that is, abolished by pretreating cells with heavy-metal chelators [43]. This effect suggests a neurotoxic effect mediated by intracellular calcium increase in astrocytes induced by Aβ. In addition, it was found that astrocytes exposed to Aβ have an increased glucose uptake and hydrogen peroxide production with no changes in intracellular antioxidants, both effects mediated by activation of the PI3K pathway [44], indicating that Aβ induces alterations of astrocytes metabolism [45] that could result in increased cytotoxicity.

Using specific deletion of the immune calcineurin/nuclear factor of activated T-cell (NFAT) pathways in astrocytes, which mediates biochemical pathways leading to astrocytes activation, it has been shown in AD animal models that activated astrocytes are responsible for cognitive and synaptic function impairment mediated by amyloid depositions [46], confirming a deleterious role of activated astrocytes in AD.

Nevertheless, even though activated astrocytes appear to have a deleterious role in AD progression, there is also evidence showing that they are capable of ameliorating the cytotoxic effects of activated microglia in culture. Conditioned media from microglia exposed to Aβ induce apoptosis in hippocampal cells, but this effect is not observed when the media is obtained from mixed glial cultures exposed to Aβ. Many of the inflammatory activation changes of microglial cell induced by Aβ are attenuated in the presence of astrocytes [47]. Moreover, astrocytes activation mediated by LPS and IFN-γ induce the secretion of TGF-β, a neuroprotective cytokine, which was capable of reducing apoptosis of hippocampal cells induced by Aβ [48], suggesting that astrocytes have a pivotal role in the modulation of AD inflammation.

Although the mechanisms that mediate astrocytes changes induced by Aβ are poorly understood, there is evidence showing that astrocytes are capable of phagocytosing Aβ and that they interact with Aβ through the SRs [23], specifically pointing out to SR-A as responsible for this interaction [35, 44, 49], which allows one to infer that, as in microglia, the phagocytic activity by astrocytes could be impaired with aging. Furthermore, given the high number of astrocytes in the brain parenchyma, even if the phagocytic activity of astrocytes appears to be less robust than that of microglia, changes in phagocytosis can be highly relevant for a decreased Aβ clearance capability, as well as impairment of the regulation of microglial cells [9].

3.3. Scavenger Receptors in AD. looseness=0 Cells mediating the immune response interact with multiple environmental compounds, and depending on receptors present on their surface, their response to those signals could be pro- or anti-inflammatory. Many pattern recognition receptors (PRR) have been described, like Toll-like receptors (TLR) and Nod-like receptors (NLR) that trigger the activation of specific inflammatory pathways according to the ligand they bind [50]. In addition, many immune cells are able to phagocytose diverse compounds because of the presence of receptors that uptake various ligands such as cell debris and allow their removal through the lysosomal pathway [51]. Many of these receptors belong to the scavenger receptors (SRs) family, a term that was first coined in 1979 to define high-affinity binding sites for acetylated low-density lipoproteins (acLDL) on macrophages [52]. These receptors share the capability of binding polyanionic ligands without differentiating exogenous ligands like those from pathogens and endogenous ligands, which have importance in the host defense response.

Whereas participation of SRs has been widely described in atherosclerosis, their role in AD-associated immune response remains poorly understood. However, it has been shown that SRs have an important role in the clearance of Aβ, and that the expression of these receptors decreases in aging brains of animal models of AD [53], situating SRs as important mediators of AD progression.

Until now 6 families of SRs have been described, named from SR-A to SR-F, but there are still 3 SRs that remain unclassified, which are RAGE, CD136, and SR-PSOX. It is unknown if the last two receptors are associated with the pathophysiology of AD, even though both are expressed in the CNS [54]. In this section we will discuss the main SRs that appear to be involved in AD and their role in glial cell inflammatory activation (Figure 1).

3.4. SR-BI. The principal ligand of SR-BI is HDL [55], which has an important role in lipid and cholesterol mobilization in the "reverse cholesterol transport" [56]. Because of the delivery of cholesterol from peripheral tissue occurs in SR-BI-expressing cells participating in lipid metabolism, the main expression of this receptor can be found in the liver and in steroidogenic tissues. Nevertheless, SR-BI can be also found in the brain parenchyma, especially on glial cells [49] and cerebral arterial smooth muscle [57].

It is believed that SR-BI has a role in host defense [58] because of the upregulation of its expression during phagocytic and dendritic differentiation of monocytes and

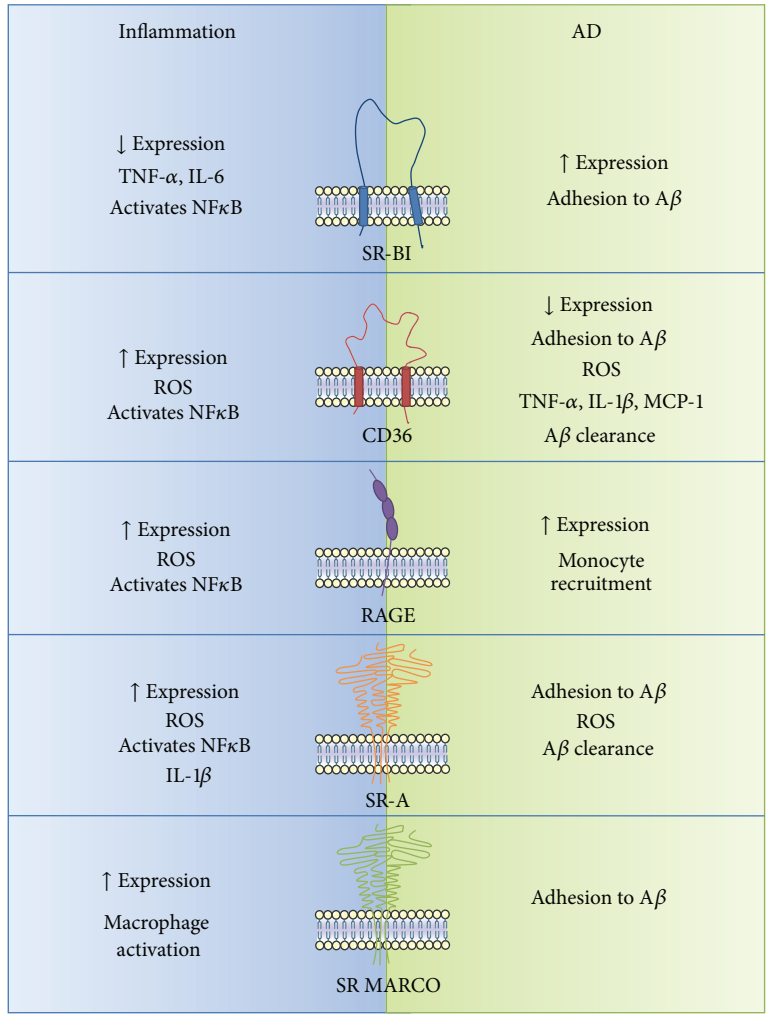

Figure 1: Summary of the main characteristics and functions played by SRs in inflammation and AD. The left panel shows changes induced by the inflammatory activation of SRs reviewed in the text. The right panel resumes the principal alterations induced by the activation of SRs mediated by the amyloid peptide.

because of the suppression of its expression in monocytes and macrophages, exposed to proinflammatory stimuli [59]. Moreover, SR-BI-null mice have a 100% fatality induced by sepsis with increased levels of inflammatory cytokines released by macrophages [60], while overexpression of SR-BI attenuated the inflammatory response in these cells [61], suggesting a protective role of SR-BI through the modulation of the inflammatory response of macrophages.

In astrocytes, the main role of SR-BI appears to be associated with clearance of apoptotic cells [62]. However, it has also been reported that SR-BI is involved in the adhesion of microglia to Aβ plaques [63]. Studies have shown that animal models for Aβ accumulation have an increased expression of SR-BI in the brain, and partial or total deletion of SR-BI gene in those animals enhances Aβ deposition associated with an impaired response of perivascular macrophages to Aβ [64]. However, if SR-BI has a direct role in Aβ clearance and if the interaction between Aβ and SR-BI activates specific signaling pathways remain as unanswered questions.

3.5. CD36. CD36, another member of the SR-B family, was initially described in adipocytes and myocardium, where it participates in the transport of long-chain fatty acids (LCFA) [65]. The deficiency of CD36 leads to an increase in plasma levels of free fatty acids and triacylglycerol, with an abnormal myocardial LCFA uptake [66]. CD36 is also expressed in immune cells, being associated with the clearance of apoptotic neutrophils by macrophages [67]. In the brain, the expression of CD36 has been reported in capillary endothelium [68], astrocytes [69], and microglial cells [70], in which this receptor has been associated with the regulation of cell migration [71].

CD36 appears to be involved in pathologies such as brain ischemia, where CD36 expression is increased mostly in cells expressing the microglia/macrophage marker CD11b. CD36-null mice have reduced infarct size after ischemia, improved neurological function, and, less ischemia-induced reactive oxygen species (ROS) levels than wild-type animals [72]. CD36-null mice also have an attenuated postischemic

activation of NFκB, suppressed glial activation [73], and an impaired astrocytes proliferation [69], all of which situate CD36 as an important mediator for brain inflammatory events.

Besides brain ischemia, Coraci et al. detected reduced levels of CD36 in microglia obtained from patients with AD, multiple sclerosis, and Parkinson's disease [70]. Specifically, in AD, CD36 appears to have a major role in the binding of cells of the monocyte/macrophage lineage to Aβ, which activates a signaling pathway associated with production of ROS and cytokines [74, 75]. In CD36-null mice, microglia and macrophages have reduced secretion of cytokines, chemokines, and ROS in response to treatment with Aβ, in addition to showing a decreased macrophage and microglial recruitment into the brain [76].

In contrast to the reports by Coraci et al. [70], Ricciarelli et al. reported high expression of CD36 in the cerebral cortex of AD patients and in normal subjects with diffuse amyloid plaques, compared with the amyloid-free brains of control individuals. Also, by using cells *in vitro*, they demonstrated that Aβ-induced CD36 overexpression in neurons was associated with an increase in nitrated proteins [77]. Nonetheless, CD36 expression by leukocytes is significantly reduced in AD patients, a phenomenon already observed at early preclinical stages as mild cognitive impairment [78].

Otherwise, in animal models of AD, it has been shown that old mice had a decreased expression of CD36 associated with an increased secretion of IL-1β and TNF-α [53], and an increased vascular amyloid deposition mediated by CD36 [79].

The ability of CD36 to participate in the clearance of Aβ has been demonstrated by Shimizu et al., who by using CHO cells overexpressing CD36, showed a dose-dependent degradation of labeled Aβ, during treatment with an anti-CD36 antibody blocked Aβ degradation [80]. In addition, astrocytes-mediated Aβ clearance is also attenuated with neutralizing antibodies against CD36 [81].

Although there are only few studies that associate CD36 with Aβ clearance, it appears that this receptor is mostly associated with neurovascular dysfunction observed in AD. In animal models of AD, deficiency of CD36 prevents cerebrovascular effects and oxidative stress elicited by Aβ [79, 82], suggesting that CD36 could be a therapeutical target mainly for the treatment of neurovascular dysfunction in AD patients.

3.6. RAGE. RAGE is a member of the immunoglobulin family and a cell surface receptor for advanced glycation endproducts (AGEs), which accumulate mainly in vascular tissues in aged individuals [83]. RAGE protein expression can be found in vasculature, endothelium, smooth muscle, mononuclear cells, cardiac myocytes, and neural tissue [84].

The interaction of RAGE with the ligand amphoterin, a polypeptide associated with the growth of cortical neurons derived from the developing brain, has been linked to cancer as the colocalization of both molecules has been shown to contribute to cellular migration and tumor invasion. Blockade of this interaction leads to suppression of the activation of intracellular pathways linked to tumor proliferation [85].

Interaction of AGEs with RAGE expressed by endothelial cells has been usually related to vascular dysfunction, mainly because of ROS induced by AGEs. This oxidative stress results in the activation of NFκB pathway [86], an affect that has been also observed in inflamed gut biopsies with a significant upregulation of RAGE [87], situating RAGE as an important inflammatory mediator in AGEs mediated lesions.

In relation to AD, the interaction of Aβ with RAGE expressed by endothelial cells of the brain favors the transendothelial migration of monocytes from peripheral blood into the brain, indicating an important role of RAGE in AD-related vascular disorders [88]. Also, an increased expression of RAGE by neurons in the brain of AD patients has been observed. Murine models of AD with overexpression of RAGE have an exacerbated impairment in spatial learning/memory and altered activation of synaptic plasticity markers [89], where the synaptic depression and LTD impairment induced by Aβ could be rescued by functional suppression of RAGE activity in microglia [90]. However, Vodopivec et al. have shown that the absence of RAGE in animal models of AD does not ameliorate their cognitive deterioration, Aβ accumulation, or microglial activation [91], suggesting that RAGE would have only a secondary role for the impairments observed in AD.

3.7. SR-A. Scavenger receptor class A (SR-A) is a homotrimeric transmembrane glycoprotein containing extracellular C-terminal cysteine-rich domains, that was initially implicated in the development of atherosclerosis, because of its colocalization with macrophages of lipid-rich atherosclerotic lesions [92, 93]. The expression of this receptor has been detected in many tissues, including liver, placenta and brain [94]. When discovered, the first function described for SR-A was to provide adhesiveness to monocytes and macrophages to glycated collagen-IV-coated surfaces, and to mediate the endocytosis of acLDL [95]. Also, given the fact that macrophage SRs are involved in the binding and internalization of LPS, which is part of Gram-negative bacteria [96], Thomas et al. showed that SR-A-deficient mice were more susceptible to infection with pathogens, with an impaired ability to clear bacterial infection, confirming what was shown by Suzuki et al., providing the first insight on the importance of SR-A in host defense [97, 98]. Additionally, other researchers observed that these animals showed a reduced expression of IL-1β, which is associated with a reduced mortality in response to LPS [99], showing that SR-A has a role in the macrophage activation induced by endotoxin shock [100–104].

In addition, SR-A also plays a role in macrophage engulfment of apoptotic thymocytes [105, 106], and in atherosclerosis, observing that disruption of the SR-A gene results in a reduction in the size of atherosclerotic lesions [98]. Moreover, in left ventricular remodeling after myocardial infarction, SR-A has a role in attenuating cardiac remodeling by suppressing macrophage polarization toward a biased

M1 phenotype, reducing the release of proinflammatory cytokines [107].

It is important that in contrast to most SRs, SR-A expression is not downregulated by chronic exposure to endogen ligands such as acLDL. On the opposite, SR-A expression can be reversibly increased by incubating macrophages with SR-A ligands [108]. In addition, binding of a ligand to SR-A activates PI3K recruits more receptors to the membrane surface [109], all of which are key functional characteristic to consider when SR-A is seen as a therapeutic target.

In the normal brain, SR-A is expressed by microglia, perivascular cells, microvessels, stromal, epiplexus, and meningeal macrophages [110–114]. Furthermore, our group was the first to describe the expression of SR-A by astrocytes, showing that exposure to SR-A ligands activated MAPKs and NFκB signaling pathways and increased the production of IL-1β and NO by astrocytes [115].

In an animal model of cerebral ischemic injury, SR-A is upregulated in the brain, an effect that correlates with increased levels of proinflammatory markers in microglia/macrophages, and an increased activation of NFκB; whereas SR-A-deficient mice show a reduced infarct size and improved neuronal function, suggesting the participation of SR-A into the M1 microglia/macrophage polarization [116–118].

In AD, SR-A has been observed in close association with senile plaques [111], presenting microglia positive for the receptor [114, 119]. SR-A has been related to rodent microglia and monocyte adhesion to Aβ coated surfaces, leading to the production of ROS [120] and to Aβ internalization mediated by endosomes in microglia [57, 121, 122]. More specifically, Aβ internalized through SR-A is trafficked toward lysosomes inside the microglia and degraded by cathepsin B [123]. To our knowledge, SR-A appears to be the most important scavenger receptor of the brain participating in Aβ clearance. For this reason, SR-A could be a potential therapeutical target in the treatment of AD.

3.8. SR MARCO. The macrophage receptor with collagenous structure (MARCO) is a member of the SRs class A family, localized mainly in macrophages of the spleen and lymph nodes [124], acting in the binding of bacterial antigens and phagocytosis, and having an important role in host defense [125]. In the presence of pro-inflammatory stimuli like LPS, SR MARCO expression is upregulated even in macrophages from liver and lungs, where normally it is not expressed [126]. It is believed that SR MARCO has a direct effect in the morphology of activated macrophages, which is necessary for trapping pathogens, by mediating the formation of lamellipodia-like structures and dendritic processes [127]. In fact, SR MARCO expression is essential for dendritic cells to acquire a mature phenotype [128].

In the CNS, SR MARCO is present in microglia and astrocytes; SR MARCO expression in microglia is associated with a decrease of the antigen internalization capacity [128], while for both astrocytes and microglial cells it is believed that SR MARCO participates in their adhesion to Aβ [49].

3.9. Other SRs in AD. There are other SRs that have been involved in the signaling mediated by Aβ, which we will briefly mention because of the scarce evidence that relates them directly to the pathophysiology of AD.

3.9.1. CD68. This receptor, also known as macrosialin, is a member of the lysosomal-associated membrane protein (LAMP) family, which is expressed in macrophages, osteoclasts, dendritic cells, and microglia, where its principal role is to bind and uptake oxidized lipoproteins and apoptotic cells [129]. Although there are no studies involving CD68 in AD, Argiles et al. showed that patients with haemodialysis-associated amyloidosis show an upregulated expression of CD68 by macrophages [130].

3.9.2. OLR1. The oxidized LDL receptor 1 (OLR1) binds LDL, being an important SR associated with atherosclerosis, is mainly expressed by endothelial cells and monocytes, with minor expression by macrophages [131]. Even though it is unknown if OLR1 is expressed by microglia, and if the receptor has a direct role in the immune response, it has been shown that OLR1 is associated with Aβ transport across the blood brain barrier [132, 133].

3.9.3. MEGF10. Multiple EGF-like domains 10 (MEGF10) is a type 1 transmembrane protein containing 17 EGF-like domains in the extracellular portion [134] and is mainly expressed in the brain, and it has been shown to be implicated in the uptake of Aβ mediated by the lipid rafts endocytosis pathway [135]. Nevertheless, MEGF10 expression has not been observed in glial cells; therefore, the mechanism by which this receptor could participate in Aβ clearance is unknown.

4. Conclusion

When AD is visualized as a pathology caused by a dysfunction of glial cells, which compromise several of their protective functions, including the phagocytosis of Aβ, and favors potentially deleterious effects, as those observed in dysregulation of the inflammatory regulation, an objective target to generate potential treatments should be the recovery of the protective activation of glia, characteristics that appear to be lost in association with aging and chronic inflammatory activation. Most of SRs found in glia appear to be potentially involved in phagocytosis and inflammatory activation of glial cells. However, they have been poorly studied in terms of their interaction with the Aβ peptide.

As it was shown by Hickman et al., the age progression associated with AD reduces the expression of SR-A in older individuals, an effect that is, also induced by treating microglial cells with pro-inflammatory cytokines [53]. This robust evidence situates SR-A as one of the main receptors involved in the impaired clearance of Aβ observed in AD and also could be the link between AD and the inflammatory state related to aging, suggesting that SR-A could be an interesting therapeutic target for AD.

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Research Article

ATP Is Required and Advances Cytokine-Induced Gap Junction Formation in Microglia In Vitro

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Microglia are the immune cells in the central nervous system. After injury microglia release bioactive molecules, including cytokines and ATP, which modify the functional state of hemichannels (HCs) and gap junction channels (GJCs), affecting the intercellular communication via extracellular and intracellular compartments, respectively. Here, we studied the role of extracellular ATP and several cytokines as modulators of the functional state of microglial HCs and GJCs using dye uptake and dye coupling techniques, respectively. In microglia and the microglia cell line EOC20, ATP advanced the TNF-α/IFN-γ-induced dye coupling, probably through the induction of IL-1β release. Moreover, TNF-α/IFN-γ, but not TNF-α plus ATP, increased dye uptake in EOC20 cells. Blockade of Cx43 and Panx1 HCs prevented dye coupling induced by TNF-α/IFN-γ, but not TNF-α plus ATP. In addition, IL-6 prevented the induction of dye coupling and HC activity induced by TNF-α/IFN-γ in EOC20 cells. Our data support the notion that extracellular ATP affects the cellular communication between microglia through autocrine and paracrine mechanisms, which might affect the timing of immune response under neuroinflammatory conditions.

1. Introduction

Microglia are the major immune effectors in the central nervous system (CNS). Under resting conditions, surveillance microglia have a ramified morphology and monitor their local microenvironment [1, 2]. However, microglia can rapidly become activated in response to diverse stimuli and danger signals, such as ATP or bacterial lipopolysaccharide (LPS) [1–3]. Consistently, microglia are activated in neuroinflammatory conditions and are a common hallmark in many neurodegenerative diseases [1, 2, 4].

Microglial cell activation includes morphological changes, proliferation, recruitment to the site of injury, and expression of specific proteins including MHC II molecules and cell adhesion molecules [1, 2]. Activated microglia also release cytokines, including TNF-α, IL-1β, IL-6, IFN-γ, and other soluble molecules, such as glutamate and ATP [5–9]. Many of these pro-inflammatory molecules act in an autocrine manner and show synergism, increasing the activation of microglia [10–12].

Many studies have focused on ATP release mechanisms and the subsequent receptors activation at the CNS, because they promote the release of other pro-inflammatory molecules, such as TNF-α and IL-1β [13]. These cytokines mediate cell communication and Ca^{2+} signaling among microglia, as well as among microglia and astrocytes [14–16]. Microglia sense extracellular ATP through P2Y and P2X receptors [1]. Under control conditions, microglia express

$P2X_7$ receptors, which are upregulated as a required step for microglial activation induced by amyloid-β peptide [17, 18]. Moreover, activation of microglia with LPS increases the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) and ATP release, through $P2X_7$ receptors [17, 19, 20]. Accordingly, cytokines that increase $[Ca^{2+}]_i$ or a calcium ionophore induce microglia activation [21, 22]. These conditions also induce gap junctional communication in primary cultures of rat or mouse microglia [23, 24].

Gap junction channels (GJCs) communicate the cytoplasm of contacting cells allowing the direct transfer of ions, second messengers, and other molecules including antigen peptides [25]. Each GJC is formed by the serial docking of two hemichannels (HCs), which are composed of six protein subunits called connexins (Cxs) [25]. It is known that resting microglia express Cxs 32, 36, 43, and 45 and after microglia activation some of them form functional GJCs and HCs [23, 24, 26–28]. Recently, another family of proteins termed pannexins (Panxs) has been found to form functional GJCs and HCs [29]. Like Cx HCs, Panx HCs are permeable to ATP and are activated by increased $[Ca^{2+}]_i$ and extracellular ATP via P2 receptors [30–32]. Microglia express functional Panx1 HCs that contribute to ATP-induced migration and glutamate and ATP release promoting neuronal death [33–35]. Under inflammatory conditions, gap junctional communication between cultured astrocytes is reduced, whereas the activity of HCs is increased [35–38]. However, it remains unknown if these opposite changes in GJCs and HCs also occur in microglia, or if extracellular ATP plays a role in this channel-based communication.

In this work, we studied the effect of extracellular ATP on the cytokine-induced gap junctional communication in microglia. To achieve this goal, we used primary cultures of rat microglia and EOC20 cells treated with several cytokines and ATP, either mixed or alone. We propose that TNF-α/IFN-γ induce gap junctional communication, which might depend on the functional expression of HCs. In addition, we found that extracellular ATP advances the onset of cytokine-induced expression of gap junctional communication, a process that was mediated by IL-1β release and inhibited by IL-6.

2. Materials and Methods

2.1. Materials. Modified Eagle's medium (MEM), Dulbecco's modified Eagle's medium (DMEM), F-12 nutrient mixture, fetal bovine serum (FBS), bovine pancreas DNAse I, and trypsin-EDTA were purchased from Gibco (Auckland, NZ, USA). DMSO, HEPES, H_2O, $LaCl_3$ (La^{3+}), ethidium (Etd) bromide, Lucifer yellow dilithium salt (LY, MW: 457,25 Da), rhodamine-dextran (RD, MW: 10 kDa), adenosine 5′-triphosphate periodate oxidized sodium salt (oATP), ATP disodium salt, probenecid (Pbc), recombinant mouse TNF-α, recombinant mouse IL-1β, recombinant mouse IFN-γ, recombinant mouse IL-6, and Ponceau S red were purchased from Sigma-Aldrich (St Louis, MO, USA). Interleukin-1 receptor antagonist (IL-1ra) was from R&D (USA). BAPTA-AM was purchased from Molecular Probes (Eugene, Oregon, USA). Penicillin, and streptomycin were obtained from Invitrogen (Carlsbad, CA, USA). D(+)-glucose, sodium hydrogen carbonate ($NaHCO_3$) were purchased from Merck (Darmstadt, Germany). ^{10}Panx1 mimetic peptide (sequence WRQAAFVDSY) was purchased from SBS Biotech (Beijing, China). Purified rat anti-mouse CD16/CD32 (mouse BD Fc-block) was purchased from BD Pharmingen (San José, CA, USA). $F(ab')_2$ fragments of a previously characterized polyclonal rabbit anti-Panx1 serum used [39, 40]. The $F(ab')_2$ fragments of affinity IgGs purified from the anti-Panx1 serum were prepared as previously described [41]. Anti-Cx43 monoclonal antibody was obtained from BD Biosciences (Minneapolis, MN, USA). Cy2 conjugated goat anti-rabbit and Cy3 conjugated goat anti-mouse antibodies were purchased from Jackson Immunoresearch Laboratories Inc. (Indianapolis, IN, USA). EDTA solution, Halt protease inhibitor single-use cocktail, and M-PER mammalian protein extraction reagent were purchased from Thermo Scientific (Rockford, IL, USA). Mount solution fluoromount G was purchased from Electron Microscopy Sciences (Washington, PA, USA). Images were examined with a confocal laser-scanning microscope which was Olympus Fluoview FV1000 (Tokio, Japan). $Cx43^{(E2)}$ is a rabbit polyclonal antibody that recognizes amino acid residues located at the second extracellular loop of Cx43 and blocks specifically Cx43 HCs [42].

Bio-Rad protein assay was purchased from Bio-Rad Laboratories (Richmond, CA, USA). SuperSignal kit for enhanced chemiluminescence detection and anti-rabbit antibody conjugated to horseradish peroxidase were purchased from Pierce (Rockford, IL, USA). EOC20 and LADMAC cells were obtained from ATCC (Manassas, VA, USA). Tissue culture flasks (25 and 75 cm^2) 60 mm and 100 mm tissue culture dishes were purchased from Sarstedt (Newton, NC, USA). Twenty four-well plastic dishes were purchased from Nunclon (Roskilde, Denmark).

2.2. Cell Cultures

2.2.1. Rat Microglia. Primary cultures of microglia were prepared from neocortex of newborn Sprague Dawley rats, as previously described [23, 24]. Briefly, meninges were carefully peeled off and cortices were dissected and minced in small pieces. After incubation in Ca^{2+}-free PBS containing trypsin (0.5%) and EDTA (5 mM) at 37°C for 30 min, tissue was triturated in presence of DNAse using a Pasteur pipette. Dissociated cells were pelleted and resuspended in MEM medium supplemented with 10% FBS, 100 units/mL penicillin and 50 μg/mL streptomycin sulfate and plated on plastic culture flasks (Sarstedt). Confluent glial cell mixed cultures were deprived of fresh medium for two weeks to induce microglial cell proliferation. Finally, microglia were harvested from glial cell mixed cultures by differential adhesion and seeded on glass coverslips.

2.2.2. EOC20 Cells. EOC20 cells are a murine microglial cell line derived from C3H/HeJ mice, which secrete cytokines and

present antigens as primary microglia [43]. EOC20 cells were maintained according to ATCC recommendations, using DMEM supplemented with 10% FBS and 20% LADMAC conditioned medium (see below). The medium was partially changed twice a week and completely changed once a week until the culture reached confluence. Cells were detached with trypsin-EDTA for 2 min and mechanical stress, and EOC20 cells were seeded on glass coverslips or tissue culture dishes. Since rat microglia were detached by shaking using an orbital shaker, some experiments were performed with EOC20 cells detached with the same methods of purification used for primary microglia cultures. No differences were observed in the induction of dye coupling between EOC20 cells obtained by the different purification methods (data not shown).

2.2.3. LADMAC Conditioned Medium. The conditioned medium was obtained from LADMAC cells, which are myeloid cells derived from murine bone marrow cells. LADMAC cells are nonadherent cells that secrete colony-stimulating-factor-1 (CSF-1) which stimulates cell division in EOC20 cells [43, 44]. LADMAC cell cultures were maintained in culture in MEM supplemented with 10% FBS during two weeks. Fresh medium was added every two days duplicating the previous amount of medium. After two weeks in culture, the cell suspension was centrifuged and the CSF-1-containing supernatant was filtered, aliquoted, and stored at −20°C until use.

2.3. Dye Transfer Technique. The transference of fluorescent dyes between adjacent cells has been used to monitor the functional state of GJCs in microglia [23, 24, 27]. We tested the intercellular transference of LY using RD as a negative control. Dyes (5% w/v in 150 mM LiCl) were microinjected by applying current to microglia seeded on glass coverslips (8×10^5 cells/well, in a 24-multiwell dish) through glass microelectrodes until the impaled cells were fluorescent. Cultures were maintained in F-12 medium supplemented with HEPES and observed with an inverted microscope equipped with Xenon arc lamp illumination and a Nikon B filter (excitation wavelength, 450–490 nm; emission wavelength, above 520 nm). Dye transfer was scored at 2 min injection. The incidence of dye coupling (IDC) was calculated as the percentage of injected cells with dye transfer to one or more neighboring cells by the total number of cells microinjected in each experiment. At least 10 cells were microinjected in each assay. Since cytokine treatments induced HC activity and because that dye uptake from leaking microelectrodes could affect the measurement of fluorescent cells, we use 200 μM La^{3+} in the recording solution. However, no significant differences were observed compared to recording solution without La^{3+} (data not shown).

2.4. Dye Uptake, Ca^{2+} Signal Imaging, and Time-Lapse Fluorescence Imaging. To evaluate dye uptake, cells seeded on glass coverslips (8×10^5 cells/mL) were exposed to 5 μM ethidium (Etd) bromide with Locke's saline solution (in mM: 154 NaCl; 5.4 KCl; 2.3 $CaCl_2$; 1 mM $MgCl_2$; 5 mM glucose; 5 mM HEPES; pH: 7.42) and examined by epifluorescence. Nuclei fluorescence was recorded in regions of interest consisting of 30 different cells per field with a water immersion Olympus 51W1I upright microscope (Melville, NY, USA), as described [45]. The calculation of slope change regression lines was fitted to points before and after treatments using Microsoft (Seattle, WA, USA) Excel. In ATP-induced dye uptake experiments, 500 μM ATP was added to recording solution after 5 min of basal dye uptake.

To evaluate Ca^{2+} signals, EOC20 cells under control conditions or after treatment were maintained as mentioned above but were loaded for 30 min with 5 μM Fura-2 AM in DMEM medium without serum at 37°C. Loaded cells were washed twice with Locke's solution and time-measurements were performed with an Olympus 51W1I microscope. The acquisition of 340 and 380 nm excitation wavelengths was every 3 s. Regions of interest consisted in 30 cells per field and analysis were performed using METAFLUOR software.

2.5. Western Blot. Confluent microglia cultures grown in 60 mm culture dishes (2.4×10^6 cells) were gently rinsed twice with cold PBS at 4°C, pH 7.4 and harvested by scraping with a rubber policeman in a solution containing 5 mM EDTA, Halt, and M-PER protein extraction cocktail according to the manufacturer's instructions. The cellular suspension was sonicated on ice. Proteins were measured in aliquots of cell lysates using the Bio-Rad protein assay. Aliquots of cell lysates (50 μg of protein) were resuspended in Laemli's sample buffer and separated in an 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis and electrotransferred to nitrocellulose sheets as previously described [24]. Loading equivalences were confirmed by protein staining with Ponceau S red (2% w/v in 30% trichloroacetic acid). Nonspecific protein binding was blocked by incubation of nitrocellulose sheets in 5% nonfat milk in PBS for 1 h at room temperature prior to overnight incubation with corresponding antibodies at 4°C. After several washes with PBS, blots were incubated with the secondary antibody conjugated to horseradish peroxidase for 45 min at room temperature. Immunoreactivity was detected by enhanced chemiluminescence using the SuperSignal kit according to the manufacturer's instructions.

2.6. Immunofluorescence. Microglia cultured on glass cover slips were fixed with 4% formaldehyde at room temperature for 30 min and washed twice with PBS. A blocking solution containing 1% IgG free BSA, 50 mM NH_4Cl, and 0.05% Triton X-100 in PBS was used to permeabilize and block unspecific reactive sites. Fc receptors were masked by incubating samples to a solution containing Fc-Block (1 : 100) for 45 min at room temperature. Panx1 and Cx43 were detected with a rabbit polyclonal anti-Panx1 $F(ab')_2$ fragments [40] and an anti-Cx43 monoclonal antibody, properly diluted with blocking solution, respectively. Cy2 conjugated goat anti-rabbit (1 : 300) and Cy3 conjugated goat anti-mouse $IgGF(ab')_2$ Igs fragments for 30 min at room temperature were used to detect bound primary antibody. Fluoromount G (Electron Microscopy Sciences, Washington, PA, USA) was used as an antifade solution to mount samples. Images

were examined with a confocal laser-scanning microscope (Olympus, Fluoview FV1000, Tokio, Japan).

2.7. IL-1β ELISA. The level of IL-1β present in the conditioned media of EOC20 cells was evaluated with the IL-1β ELISA Ready.Set-Go! (e-Bioscience, San Diego, CA, USA), for performing quantitative enzyme linked immunosorbent assays (ELISA). It has a sensitivity of 8 pg/mL. Standard curve consisted of twofold serial dilutions of the recombinant cytokine. In brief, a 96-well, flat bottom, ELISA-plate (MICROLON, Greiner Bio-One) was coated with capture antibody in coating buffer overnight at 4°C. The plate was washed 5 times with PBS-0.05% Tween-20 in ELx50 Biokit, a 96-well bioelisa washer, and Rhe plate was blocked with 200 μL of assay diluent at room temperature for 1 h, washed as mentioned, and 100 μL of standard IL1β and samples were incubated at 4°C overnight. Then, the plate was washed and 100 μL of detection antibody for IL-1β was added and incubated at room temperature by 1 h, washed 5 times, incubated with 100 μL Avidin-HRP at room temperature for 30 min, washed 7 times, added 100 μL substrate solution, and stopped the reaction with 50 μL of 1 M H_3PO_4. The plate was read at 450 nm, with reference at 570 nm.

2.8. Treatments. Microglia were seeded 48 h before dye transfer, dye uptake, or immunofluorescence experiments in 24-well plastic dish containing 500 μL of culture medium. For Western blot experiments, cells were seeded in 60 mm plastic dishes in 3 mL of culture medium. After 48 h under control conditions microglia were treated with 1 mM ATP or 1 ng/mL TNF-α, IFN-γ, IL-1β either alone or mixed. Cytokines were added simultaneously and ATP was added 2 h before measurement and is referred as cytokine(s) plus ATP. Treatment with 1, 10, or 50 ng/mL IL-6, 20 ng/mL IL-1ra, 300 μM oATP, 200 μM La^{3+}, 1 : 500 $Cx43^{(E2)}$ antibody or 200 μM ^{10}Panx1 was simultaneous to cytokine treatment. We used 50 μM of β-GA for acute GJCs blocking (Figure S1, see Supplementary Materials available online at http://dx.doi.org/10.1155/2013/216402). To avoid disruption of cell adhesion with BAPTA, the medium was replaced with culture medium of parallel cultures treated at the same time to maintain the soluble factor released from microglia.

2.9. Statistical Analysis. Data are presented as mean ± SEM, as percentage of the control condition; n represents the number of independent experiments. For statistical analysis, each treatment was compared with its respective control and significance was determined using one-way ANOVA followed by Dunn's test comparing all treatments against the control condition. To observe differences between microglia and EOC20 cells responses we used a two-way ANOVA.

3. Results

3.1. The Onset of the Cytokine-Induced Increase in Gap Junctional Communication in Cultured Microglia Is Advanced by ATP and Delayed by IFN-γ. Calcium ionophore and pro-inflammatory molecules promote a transient expression of functional GJCs in microglia [23, 24, 27]. Since extracellular ATP, TNF-α, and IFN-γ play a relevant role in microglial cell responses [3, 7, 46] and affect the $[Ca^{2+}]_i$ [47–49], we decided to evaluate if these compounds affect the intercellular communication via GJCs in both primary cultures of rat microglia and EOC20 cells.

After 48 h of subculture under control conditions, microglia were treated as indicated in Methods (Figure S1a). Both cell types presented rather homogeneous morphological features (Figures 1(a) and 1(b)) and very low incidence of Lucifer yellow (LY) transfer to neighboring cells (Figures 1(a) and 1(b)). Under these conditions, the incidence of dye coupling (I.D.C) remained low for up to 12 h of culture in both cell types (Figure 1, Supporting information Table S1). In addition, intercellular transfer of rhodamine-dextran (RD, ~10 kDa), which due to its high molecular weight cannot permeate through GJCs, was not observed (Figure S2a). This result indicates that intercellular LY transfer ocurred via GJCs and not through other cell-cell communication pathway, such as cytoplasmic bridges. Moreover, microglia treated either with 1 mM ATP, 1 ng/mL TNF-α, 1 ng/mL IFN-γ, or 1 ng/mL IL-1β showed only a slight increase in IDC, which was not statistically different from that of control cells ($P > 0.05$: Supporting information Table S1). However, treatment with mixes of these molecules during different time periods caused a significant and transient increase in IDC; the dye transfer data is expressed as percentage of the corresponding control condition (Figures 1(e) and 1(f)). In both cell types, treatment with 1 ng/mL TNF-α plus 1 ng/mL IFN-γ (from now and on referred as TNF-α/IFN-γ) increased the IDC, reaching a maximum response at around 9 h after treatment (IDC in EOC20 cells: 574 ± 36% of control; rat microglia, 552 ± 36% of control; Mean ± SEM; $n = 5$) as previously described [23].

We also studied if extracellular ATP affects TNF-α/IFN-γ-induced dye coupling. To this end, cells were treated with these cytokines and then exposed to ATP for 2 h. In both cell types, treatment with TNF-α/IFN-γ plus ATP induced a transient increase in IDC, which was maximal at around 5 h (EOC20 cells: 517 ± 94% of control; rat microglia: 506 ± 42% of control, $n = 5$). The amplitude and duration (magnitude) of the response was similar to that induced by TNF-α/IFN-γ, but occurred 4 h earlier (Figure 1(e)).

Since IFN-γ potentiates TNF-α-induced dye coupling in antigen presenting cells, including dendritic cells, microglia and monocytes/macrophages [23, 50, 51], we tested whether ATP induces a similar effect on microglia. In agreement with this possibility, cells treated with TNF-α plus ATP showed maximal IDC with similar amplitude (EOC20 cells: 529±12% of control; rat microglia: 534±70% of control; $n = 6$; Figure 1) to that induced by TNF-α/IFN-γ plus ATP, but occurred 1.5 h earlier (at ~3.5 h versus 5 h; Figures 1(e) and 1(f)). As mentioned before intercellular transfer of LY was enhanced in primary microglia or EOC20 cells treated with TNF-α plus ATP (Figures 1(c) and 1(d)). However, intercellular transfer of RD was not observed, ruling out the formation of cytoplasmic bridges or vesicular mediated dye transfer in each condition (Figure S2). Microglia treated with IFN-γ plus ATP did not increase dye coupling at 3.5 h (EOC20 cells: 167 ± 97% of

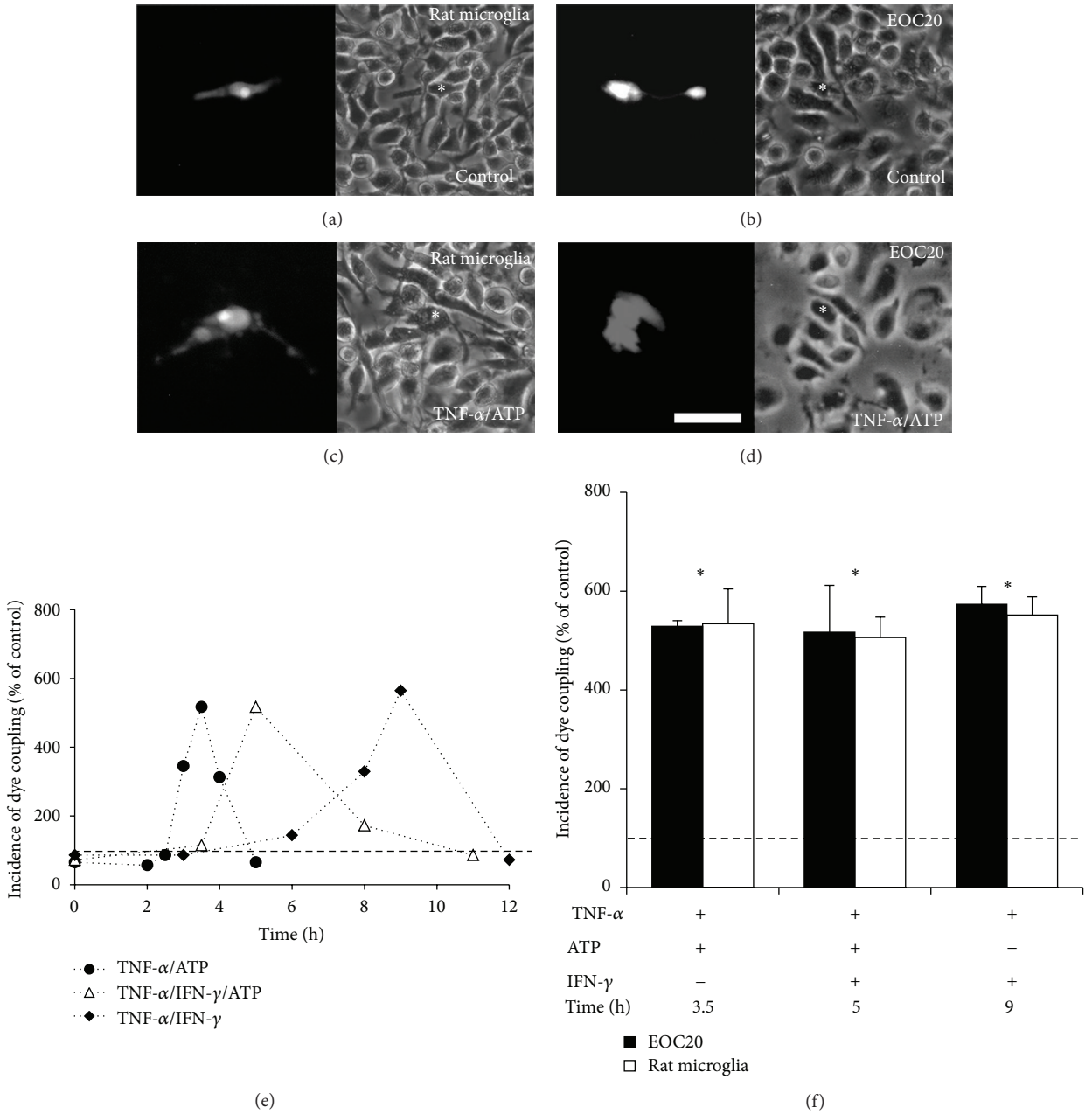

Figure 1: ATP advances the onset of TNF-α/IFN-γ-induced dye coupling. (a)–(d) Dye transfer was evaluated 2 min after Lucifer yellow (LY) microinjection in a single cell (indicated with an asterisk). Representative pictures of LY transfer in rat microglia (a), (c) or EOC20 cells (b), (d) under control condition or after TNF-α plus ATP (TNF-α/ATP) treatment for 3.5 h, as indicated. Phase contrasts of each micrograph are shown at the right panels. Scale bar: 20 μm. (e) Time course of the incidence of dye coupling (IDC) as percentage of IDC in EOC20 cells under control conditions (dashed line) or after treatment with TNF-α plus ATP (black circles), TNF-α/IFN-γ plus ATP (white triangles), or TNF-α/IFN-γ (black diamonds). Each point corresponds to the mean of 3 independent experiments. (f) Graph showing the maximum values of IDC after treatment with TNF-α plus ATP for 3.5 h, TNF-α/IFN-γ plus ATP for 5 h, or TNF-α/IFN-γ for 9 h. $^*P < 0.05$ versus control condition. Each bar represents the mean ± SEM, $n = 6$. No significant differences were observed when comparing microglia and EOC20 cells responses to different treatment in dye transfer assays. Concentrations: 1 ng/mL TNF-α; 1 mM ATP; 1 ng/mL IFN-γ.

control; rat microglia: 210.8±51.3% of control) or other times (2 and 5 h, data not shown).

3.2. The Increase of Gap Junctional Communication Induced by TNF-α Plus ATP Requires an Increase of $[Ca^{2+}]_i$ via Activation of P2X Receptors and Is Prevented by IL-6. Eugenín et al. (2001) described that dye coupling between microglia treated for 9 h with TNF-α/IFN-γ is inhibited by β-GA. In EOC20 cells treated with TNF-α/IFN-γ, we observed a similar acute blockade with β-GA (data not shown).

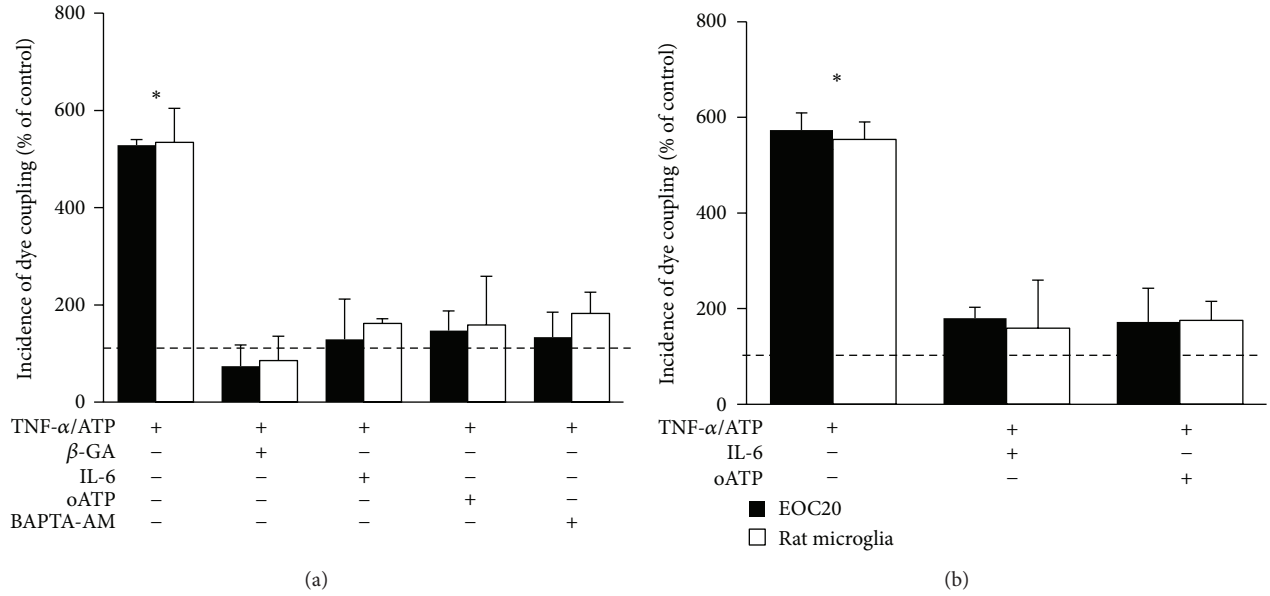

Figure 2: IL-6, intracellular calcium chelation, and P2X inhibition prevent the induction of gap junctional communication promoted by TNF-α plus ATP or TNF-α/IFN-γ. (a) Graph showing the effect of acutely applied 50 μM 18-β-glycyrrhetinic acid (β-GA) or pretreatment with 10 ng/mL interleukin-6 (IL-6), 300 μM oxidized ATP (oATP), or 10 μM BAPTA-AM on the incidence of dye coupling (IDC) of microglia treated for 3.5 h with TNF-α plus ATP. (b) Graph showing the effect of 50 ng/mL IL-6 or 300 μM oATP over the IDC of microglia treated for 9 h with TNF-α/IFN-γ. Data is expressed as a percentage of IDC under control conditions (dashed line). $^*P < 0.05$ versus control condition. Each bar represents the mean ± SEM, $n = 5$. No significant differences were observed when comparing microglia and EOC20 cells responses to different treatment in dye transfer assays.

In addition, application of 50 μM β-GA for 5 min completely abolished dye coupling induced by TNF-α plus ATP (IDC in EOC20 cells: 74 ± 44% of control; rat microglia: 86 ± 50% of control; $n = 5$; Figure 2(a)).

Since microglia treated with purinergic agonists release IL-6 [52], and this cytokine prevents the increase of dye coupling induced by TNF-α/IL-1β in dendritic cells [50], we decided to test if IL-6 prevents induction of dye coupling in microglia treated with TNF-α plus ATP. In cell cultures treated simultaneously with 10 ng/mL IL-6 plus TNF-α and then treated with ATP for 3.5 h, the IDC was low (EOC20 cells: 130 ± 83% of control; rat microglia: 162 ± 10% of control; $n = 4$) similar to the results obtained under control conditions (Figure 2(a)). Similarly, the TNF-α/IFN-γ-induced dye coupling was prevented by IL-6 (Figure 2(b)). This inhibitory effect was IL-6 concentration-dependent (1, 10, and 50 ng/mL, data not shown). The maximal effect was induced by 50 ng/mL IL-6 (EOC20: 180 ± 23% of control; rat microglia: 159 ± 100% of control; $n = 4$; Figure 2(b)).

Since microglia express several P2X and P2Y receptors [3], the possible involvement of purinergic receptors in the TNF-α/IFN-γ-induced dye coupling in microglia treated with oxidized-ATP (oATP), an inhibitor of P2X receptors [53], was studied. Coapplication of 300 μM oATP prevented dye transfer induced by TNF-α plus ATP (IDC in EOC20 cells: 147±41% of control; rat microglia: 159±100% of control; $n = 5$; Figure 2(a)) or by TNF-α/IFN-γ (IDC in EOC20: 172 ± 70% of control; rat microglia: 176 ± 40% of control; $n = 5$; Figure 2(b)). Moreover, cells treated with TNF-α plus 1 mM ADP, a P2Y agonist [53], for 3.5 h did not show changes in dye coupling (IDC in EOC20 cells: 168 ± 84% of control, $n = 3$), suggesting that P2Y receptors are not involved in ATP-induced gap junctional communication in microglia.

Since activation of P2 receptors promotes a rise in $[Ca^{2+}]_i$ in microglia [54], we tested if this response was related to the increase in dye coupling induced by TNF-α plus ATP. Cells were loaded with BAPTA, a Ca^{2+} chelator, and then washed and the extracellular medium was replaced with conditioned medium of cultures treated in parallel with TNF-α for 90 min to maintain the culture conditions as before loading with BAPTA. In these cells, treatment with TNF-α plus ATP did not increase dye coupling (IDC in EOC20 cells: 134 ± 51% of control; rat microglia: 183 ± 44% of control; $n = 5$; Figure 2(a)). In addition, we observed that EOC20 cells treated with TNF-α plus ATP present increased Ca^{2+} signal, compared to cells under control conditions (Figure S3a). Interestingly, IL-6 prevented this rise in the Ca^{2+} signal (Figure S3b), suggesting that IL-6 might regulate the purinergic signaling in EOC20 cells.

3.3. IL-1β Released by Activated Microglia Mediates the TNF-α/IFN-γ-Induced Dye Coupling in EOC20 Cells. Since activated microglia release IL-1β and its natural antagonist IL-1ra [7, 55], we studied possible involvement of these molecules in the transient increase in dye coupling induced by TNF-α plus ATP or TNF-α/IFN-γ. Coapplication of 20 ng/mL IL-1ra significantly prevented the increase in IDC induced by TNF-α plus ATP (in EOC20 cells: 217 ± 36% of control, $n = 4$) or TNF-α/IFN-γ (in EOC20 cells: 241 ± 53% of control, $n = 4$;

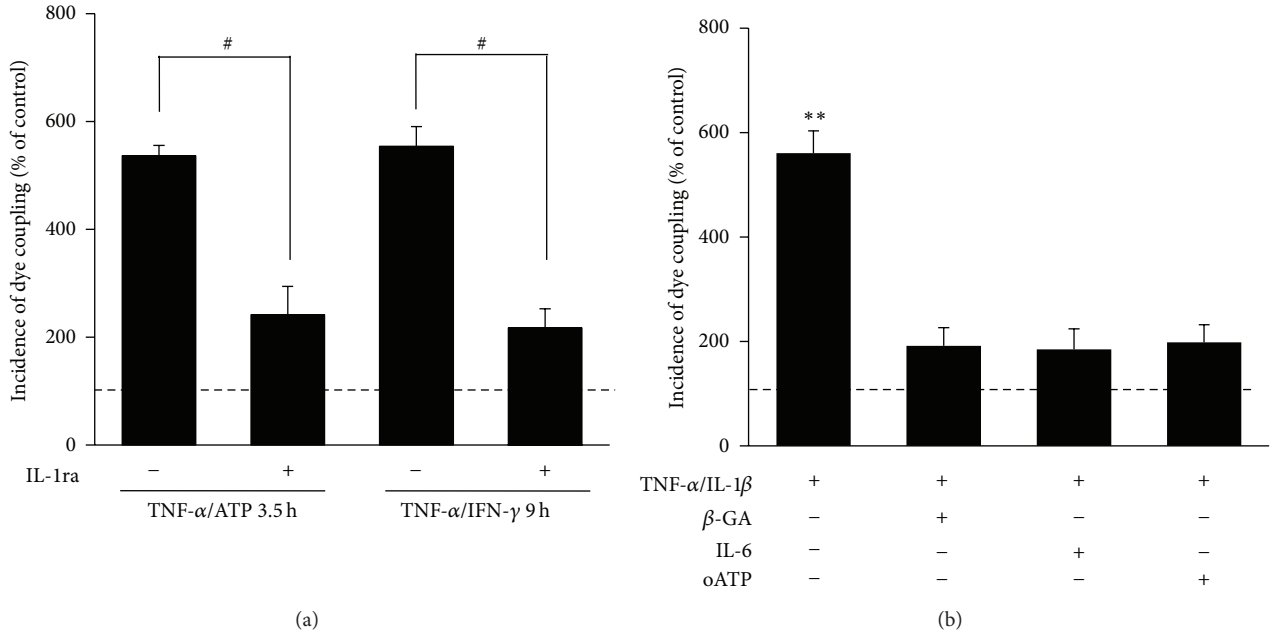

Figure 3: IL-1β mediates gap junctional communication induced by pro-inflammatory molecules. (a) Effect of 20 ng/mL IL-1 receptor agonist (IL-1ra) over LY transfer in EOC20 cells treated with TNF-α plus ATP for 3.5 h or with TNF-α/IFN-γ for 9 h. $^{\#}P < 0.05$ between indicated treatments. Each bar represents the mean ± SEM, $n = 4$. (b) The effect of 50 μM 18-β-glycyrrhetinic acid (β-GA) acutely applied or treatment with 10 ng/mL interleukin-6 (IL-6) or 300 μM oxidized ATP (oATP) over LY transfer in EOC20 cells treated with TNF-α/IL-1β for 9 h is also shown. Each bar represents the mean ± SEM, $n = 4$, and corresponds to the percentage of incidence of dye coupling under control conditions (dashed line). $^{*}P < 0.05$, $^{**}P < 0.01$ versus control condition.

Figure 3(a)). Moreover, EOC20 cells showed an increase in IL-1β release after TNF-α plus ATP or TNF-α/IFN-γ stimulation, which was partially prevented by IL-6 (Figure S4). Consistent with the involvement of IL-1β in the above dye coupling response induced by both pro-inflammatory molecules, exogenous application of 1 ng/mL IL-1β plus TNF-α induced a similar response than that elicited by TNF-α plus ATP or TNF-α/IFN-γ (Figure 3(b)). EOC20 cells treated with TNF-α/IL-1β showed a transient increase in dye coupling (data not shown), reaching a maximal IDC at ~9 h of treatment (EOC20 cells: 560 ± 43% of control, $n = 4$; Figure 3(b)). The TNF-α/IL-1β-induced dye coupling was drastically reduced by the acute application of 50 μM β-GA (IDC in EOC20 cells: 192 ± 35% of control, $n = 4$) and prevented by 10 ng/mL IL-6 (in EOC20 cells: 185 ± 40% of control, $n = 4$) or 300 μM oATP (in EOC20 cells: 198 ± 34% of control, $n = 4$) coapplied with the two cytokines (Figure 3(b)). However, treatment with IL-1β did not increase dye coupling in EOC20 cells (data not shown).

3.4. TNF-α/IFN-γ but Not TNF-α Plus ATP Increases Plasma Membrane Permeability in EOC20 Cells. Astrocytes treated with TNF-α/IL-1β for 24 h [38] and microglia treated with LPS (or TNF-α) for 24 h showed an increased HC activity [28, 35, 56–58]. Using the ethidium (Etd) uptake assay to evaluate the functional state of HCs located at the cell surface [38, 59], we studied if TNF-α or ATP affects the membrane permeability of microglia cells. In EOC20 cells, Etd uptake evaluated with time-lapse measurements showed no significant differences after treatment with TNF-α plus ATP as compared to untreated cells (Figure S5). In control conditions, Etd uptake was partially blocked by 200 μM La^{3+} (after La^{3+}: 45 ± 11% of control, $n = 5$), a Cx HC blocker that does not affect Panx HCs [31] and by 10 μM carbenoxolone (Cbx) (after Cbx: 36 ± 15% of control, $n = 5$), which at this concentration inhibits mainly Panx HCs [60]. A slight, but not statistically significant increase in Etd uptake was recorded after 3.5 h treatment with TNF-α plus ATP (134 ± 25% of control, $n = 5$) and was inhibited by La^{3+} (after La^{3+}: 47 ± 8% of control, $n = 5$) or Cbx (after Cbx: 38 ± 8% of control, $n = 5$), suggesting an upstream cross talk between Cx and Panx HCs. In addition, 10 ng/mL IL-6 did not affect the response induced by TNF-α plus ATP treatment for 3.5 h (Etd uptake rate: 141 ± 16% of control, $n = 5$; Figure S5b). In contrast, after treatment with TNF-α/IFN-γ for 9 h, a statistically significant increase in the Etd uptake rate compared to the control condition was detected (Figure 4). In EOC20 cells cultured for 9 h under control conditions the Etd uptake rate remained low and was partially blocked by La^{3+} (57 ± 17% of control, $n = 5$; Figures 4(a) and 4(e)) or Cbx (34 ± 4% of control, $n = 5$; Figure 4(e)). However, cells treated with TNF-α/IFN-γ for 9 h showed a prominent increase in Etd uptake (237 ± 25% of control, $n = 5$) that was drastically reduced by La^{3+} (51 ± 12% of control, $n = 5$; Figures 4(a) and 4(e)) or Cbx (76 ± 9% of control, $n = 5$; Figure 4(e)). A similar increase in dye uptake was found after treatment with TNF-α/IL-1β for 9 h (Etd uptake rate: 197 ± 41% of control, $n = 3$), which was also reduced by La^{3+} (Etd uptake rate: 105 ± 4%

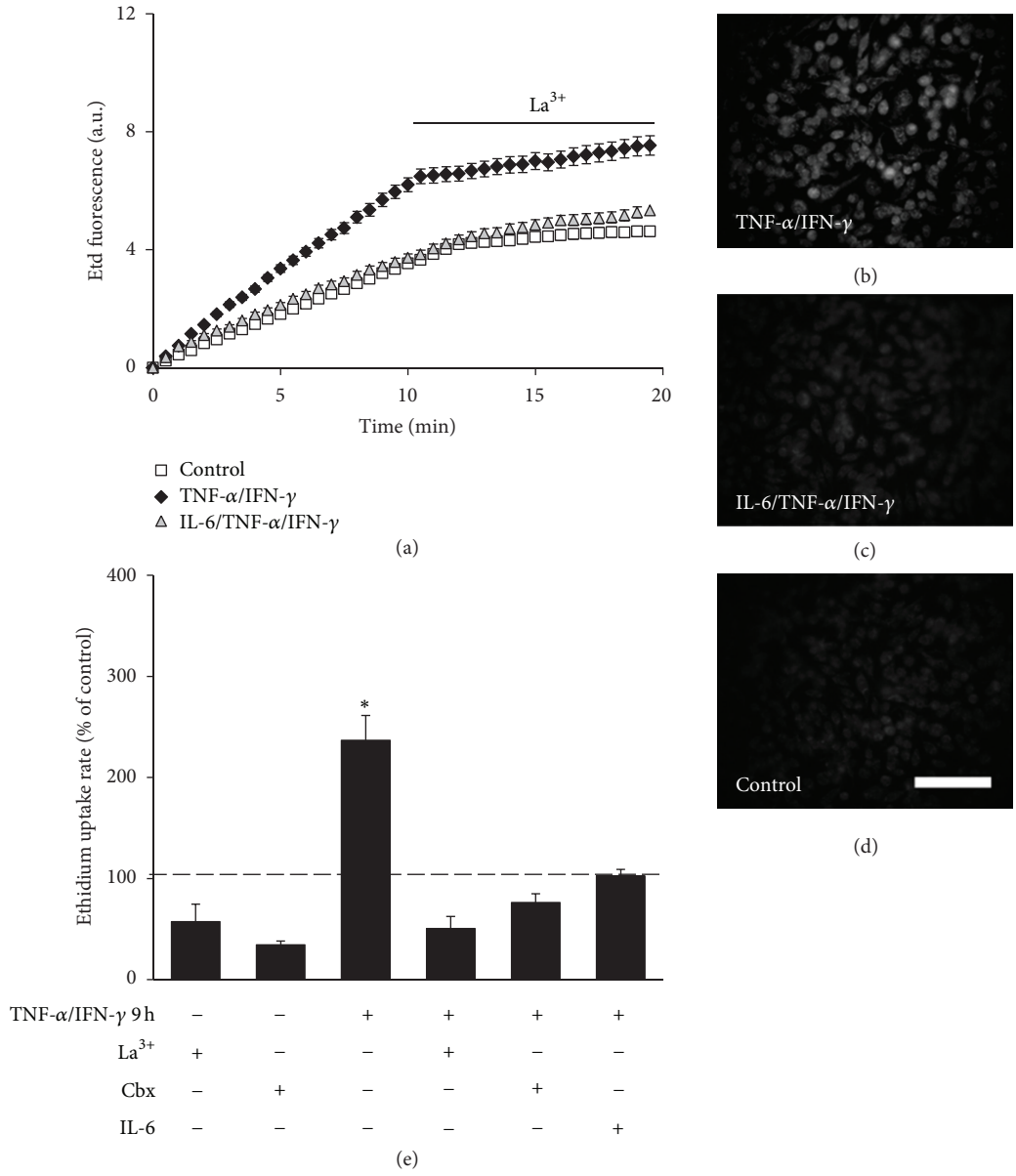

Figure 4: TNF-α/IFN-γ induces membrane permeabilization in EOC20 cells. (a) Time-lapse measurements of ethidium (Etd) uptake in EOC20 cells under control conditions (white squares), or after treatment with TNF-α/IFN-γ (black diamonds) or IL-6/TNF-α/IFN-γ (gray triangles) for 9 h. Each value represents the mean ± SEM of 30 cells. After 10 min of basal uptake, 200 μM La^{3+} was applied to the bath. (b)–(d) Representative fluorescence micrographs of Etd uptake after 10 min of Etd uptake under indicated treatments, previous to La^{3+} application. Scale bar: 100 μm. (e) Graph showing the acute effect of 200 μM La^{3+}, 10 μM carbenoxolone (Cbx), or pretreatment with 50 ng/mL of interleukin-6 (IL-6) Etd uptake rate expressed as percentage of control conditions (dashed line) in EOC20 cells treated with TNF-α/IFN-γ for 9 h. Each bar corresponds to the mean ± SEM, $n = 5$. $^{*}P < 0.05$ versus control condition.

of control, $n = 3$). Moreover, coapplication of 50 ng/mL IL-6 with TNF-α/IFN-γ prevented the Etd uptake rate increase in cells treated just with TNF-α/IFN-γ (96 ± 67% of control, $n = 5$; Figure 4(e)). In the latter conditions, the Etd uptake rate was slightly reduced by La^{3+} (48 ± 8% of control, $n = 5$).

3.5. Extracellular ATP Increases the Plasma Membrane Permeability in EOC20 Cells. Extracellular ATP, in the millimolar range, induces membrane permeabilization in many cell types, including microglia [61, 62]. Similarly, ATP permeabilizes macrophages in a Panx1-dependent way [31]. We tested the effect of 2 mM ATP on Etd uptake in EOC20 cells, as previously observed in macrophages and described by others [31, 63]. A rapid increase in Etd uptake rate (expressed as % of control) was induced by the acute application of 2 mM ATP (529 ± 84% of basal uptake, $n = 5$) to cells cultured for 3.5 h under control conditions (Figures 5(a) and 5(b)). This response was drastically blocked by 10 μM Cbx (218 ± 81% of basal uptake, $n = 5$; Figure 5(a)), as well as by 50 μM β-GA,

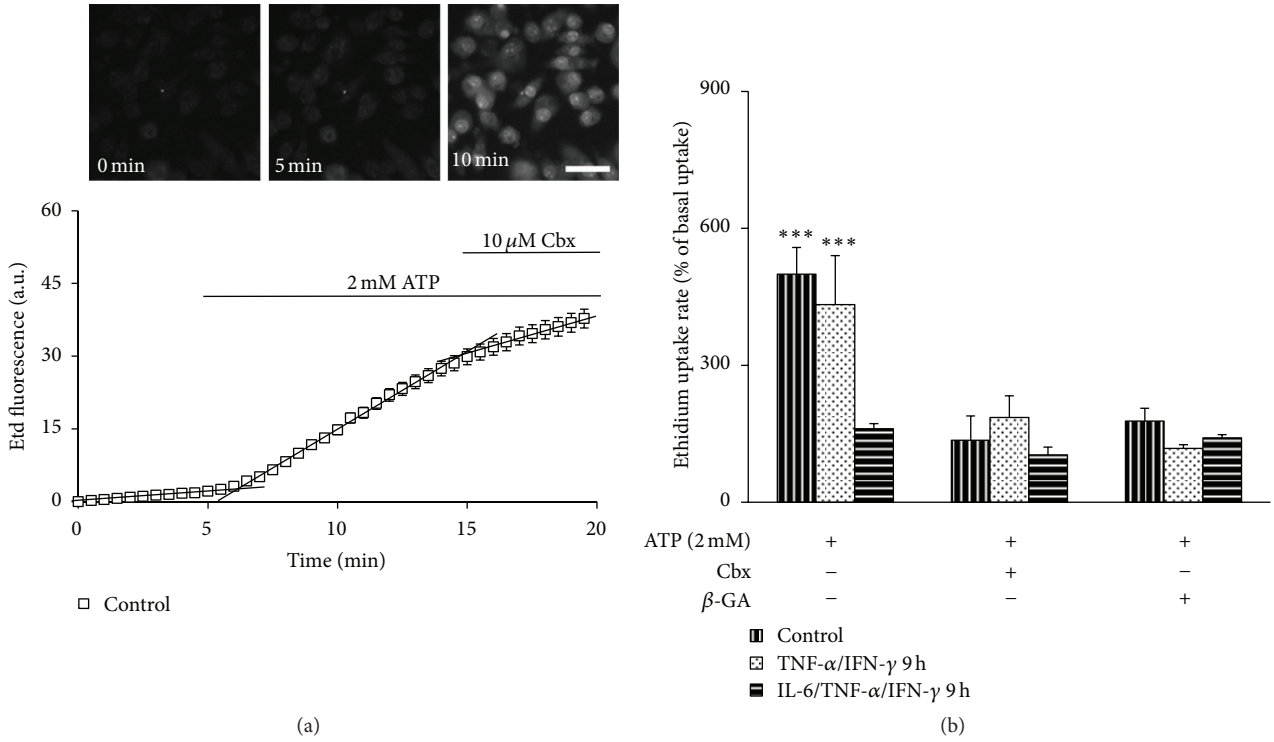

Figure 5: Extracellular ATP increases the cell membrane permeability in EOC20 cells. (a) Fluorescent views of Etd uptake of EOC20 cells cultured under control conditions before (0 min) or after application of 2 mM ATP (5 and 10 min). Scale bar: 25 μm. Time-lapse measurement. After 5 min of basal uptake, 2 mM ATP was added to extracellular solution. At 15 min of recording, 10 μM of carbenoxolone (Cbx) a HC blocker was added to the bath. Black lines denote the slope at different times of Etd uptake. Data represent the mean ± SEM of 30 cells in each of 5 independent experiments. (b) Graph showing the effect of acute application of extracellular ATP in EOC20 cells under control conditions or after treatment with TNF-α/IFN-γ or with 50 ng/mL IL-6 plus TNF-α/IFN-γ for 9 h. The effect of acute blockade with 10 μM carbenoxolone (Cbx) or 50 μM 18-β-glycyrrhetinic acid (β-GA) is also shown. Data was normalized to basal uptake in each condition (dashed line) and represents the mean ± SEM. $^{***}P < 0.001$ versus control condition.

a Cx and Panx HC blocker (128 ± 47% of basal uptake, $n = 5$; Figure 5). In cell cultures treated with TNF-α plus ATP for 3.5 h, acute treatment with ATP did not induce a statistically significant increase in Etd uptake (173 ± 17% of basal uptake, $n = 5$, Figure S6a) and was blocked by Cbx (85 ± 16% of basal uptake, $n = 5$) or β-GA (102 ± 63% of basal uptake, $n = 5$ Figure S6b). Similarly, cells treated with 10 ng/mL IL-6/TNF-α plus ATP showed a small increase in Etd uptake rate after acute application of 2 mM ATP (196 ± 28% of basal uptake, $n = 5$, Figure S6b). This response was blocked by Cbx (85 ± 28% of basal uptake, $n = 5$) or β-GA (102 ± 63% of basal uptake, $n = 5$; Figure S6b).

Moreover, EOC20 cells cultured for 9 h under control conditions showed a rapid increase of Etd uptake in response to 2 mM ATP (500 ± 58% of basal uptake, $n = 5$), which was completely blocked by Cbx (136 ± 53% of basal uptake, $n = 5$) or β-GA (178 ± 28% of basal uptake, $n = 5$; Figure 5(b)). EOC20 cells treated with TNF-α/IFN-γ for 9 h exhibited a significant increase in Etd uptake rate after ATP treatment (433 ± 107% of basal uptake, $n = 5$), which was blocked by Cbx (186 ± 47% of basal uptake, $n = 5$) or β-GA (118 ± 8% of basal uptake, $n = 5$). In contrast, in EOC20 cells treated for 9 h with 50 ng/mL IL-6 plus TNF-α/IFN-γ, ATP did not increase Etd uptake (161 ± 11% of basal uptake, $n = 5$), and neither Cbx (104 ± 17% of basal uptake, $n = 5$) nor β-GA (141 ± 7% of basal uptake, $n = 5$; Figure 5(b)) affected it.

In addition, cultures treated for 9 h with TNF-α/IL-1β showed increased Etd uptake rate after ATP application (510± 58% of basal uptake, $n = 5$, Figure S7a), which was partially blocked by Cbx (229 ± 32% of basal uptake, $n = 5$, Figure S7a) or β-GA (282 ± 35% of basal uptake, $n = 5$). Interestingly, the ATP-induced increase in Etd uptake was almost completely absent in cells pretreated with 10 ng/mL IL-6 plus TNF-α/IL-1β (243 ± 56% of basal uptake, $n = 5$, Figure S7a) and the activity present was blocked by 10 μM Cbx (210 ± 71% of basal uptake, $n = 5$) or β-GA (175 ± 49% of basal uptake, $n = 5$; Figure S7a).

3.6. Blockade of Hemichannels Reduces the TNF-α/IFN-γ-Induced Dye Coupling. Open HCs allow the release of molecules such as ATP and glutamate [35, 56–58, 64] and uptake of small molecules such as glucose [38]. In addition, in other cellular systems, functional Cx46 HCs stimulate formation of GJCs [65]. Thus, we studied the possible contribution of increased HC activity on dye coupling induced by pro-inflammatory molecules in cells incubated with HC blockers.

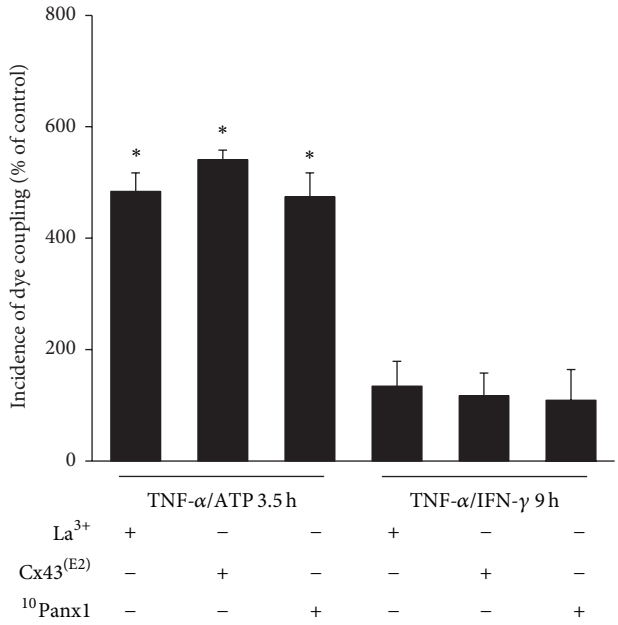

Figure 6: Blockade of HCs prevents upregulation of microglial gap junctional communication induced by TNF-α/IFN-γ, but not that induced by TNF-α plus ATP. Effect on the incidence of dye coupling (IDC) in EOC20 cells treated with TNF-α plus ATP for 3.5 h or TNF-α/IFN-γ for 9 h in presence of HC blockers (200 μM La^{3+}, 1 : 500 $Cx43^{(E2)}$ antibody, 200 μM ^{10}Panx1). Data is expressed as percentage of IDC under control conditions (dashed line) and corresponds to the mean ± SEM, $n = 4$. $^{*}P < 0.05$ versus control condition.

Treatment with 200 μM La^{3+} prevented the TNF-α/IFN-γ-induced dye coupling recorded as IDC (134 ± 45% of control, $n = 4$; Figure 6). A similar inhibitory effect was induced by the application of 1 : 500 $Cx43^{(E2)}$ antibody (117 ± 41% of control, $n = 4$), a specific Cx43 HC blocker [66], or 200 μM ^{10}Panx1 (IDC in EOC20 cells: 109 ± 55% of control, $n = 4$; Figure 6). However, neither irrelevant IgG nor scramble ^{10}Panx1 peptide prevented the TNF-α/IFN-γ-induced dye coupling (data not shown). On the other hand, treatment with La^{3+} (484 ± 34% of control, $n = 4$), $Cx43^{(E2)}$ antibody (540.8 ± 30% of control, $n = 4$) or ^{10}Panx1 (474 ± 43% of control, $n = 4$) did not change the dye coupling induced by TNF-α plus ATP (Figure 6).

3.7. Pro-Inflammatory Molecules Regulate Cx43 and Panx1 Levels and Distribution in Microglia. Cx32, Cx36, and Cx43 have been detected in cultured microglia [23, 24, 26–28]. However, Cx43 seems to be the main contributor involved in cytokine-induced gap junctional communication, because microglia from $Cx43^{del/del}$ mice do not express functional GJCs in response to TNF-α/IFN-γ [23]. In addition, expression of Panx1 by microglia has been reported recently [35]. Thus, the distribution and levels of Cx43 and Panx1 during treatments that affect GJC and HC activity were evaluated by immunofluorescence and Western blot analyses.

Under control conditions, rat microglia presented low and heterogeneous Cx43 and Panx1 reactivity (Figure 7(a)). After treatment with TNF-α plus ATP (3.5 h) or TNF-α/IFN-γ (9 h) Cx43 and Panx1 reactivity were higher than in control conditions (Figure 7(a)). However, treatment with IL-6 (10 ng/mL)/TNF-α plus ATP or IL-6 (50 ng/mL)/TNF-α/IFN-γ did not affect the reactivity of Cx43 and Panx1 (Figure 7(a)). Moreover, in cultures treated with IL-6 plus TNF-α/ATP a redistribution of Cx43 and Panx1 was observed; these proteins were segregated providing a "cell polarization" appearance, which was quantified (Figure 7(b)). Under control conditions rat microglia exhibited little or no segregation (polarized: 19 ± 6%, $n = 5$) although some cells showed more Cx43 or Panx1 reactivity. Segregation of these proteins was not significantly affected by TNF-α plus ATP for 3.5 h (polarized: 8 ± 4%, $n = 5$), but the number of cells with segregation was increased by the simultaneous treatment with IL-6 and TNF-α plus ATP (polarized: 61 ± 1%, $n = 5$). However, treatment with TNF-α/IFN-γ for 9 h did not affect the resting distribution (polarized: 21 ± 6%, $n = 5$) and remained unchanged in cells simultaneously treated with IL-6/TNF-α/IFN-γ (polarized: 15 ± 4%, $n = 5$). Similar results were found in EOC20 cells treated with TNF-α/IL-1β for 9 h (Figure S7b).

Protein levels were evaluated in EOC20 cells by Western blot analyses. Total levels of Cx43 and Panx1 increased after treatments with TNF-α plus ATP, TNF-α/IFN-γ or TNF-α/IL-1β, which caused the maximal effect on gap junctional communication (Figure 7(c)). Only the increase in total Cx43 levels was prevented by IL-6 in the same conditions that prevented the induction of dye coupling. Even when IL-6 prevented the increase in total Panx1 levels after treatment with TNF-α/IFN-γ, or TNF-α/IL-1β, coapplication of IL-6 failed to prevent the increase observed after TNF-α plus ATP treatment (Figure 7(c)).

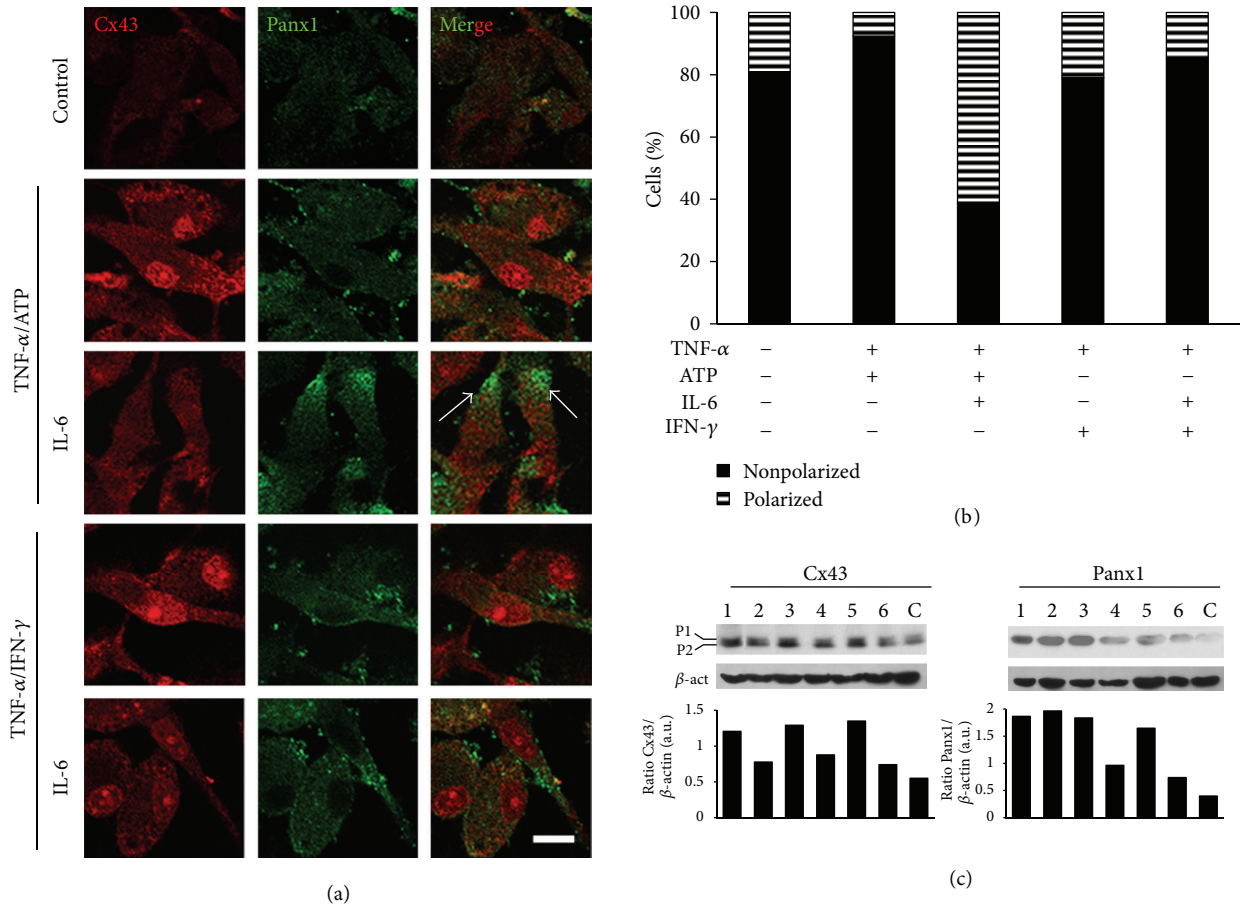

FIGURE 7: Pro-inflammatory treatments upregulate Cx43 and Panx1 protein levels in microglia. (a) Confocal images show immunoreactivity for Cx43 (red) and Panx1 (green) in primary rat microglia under control conditions of after treatment with TNF-α plus ATP for 3.5 h or with TNF-α/IFN-γ for 9 h, in absence or presence of IL-6 (10 or 50 ng/mL, respectively). Arrows show microglia with segregation of Cx43 and Panx1. Scale bar: 10 μm. (b) Quantification of nonpolarized (black bars) versus polarized (dashed white bars) rat microglia under control conditions or after treatments shown in (a). Data are expressed as a percentage of the total number of cells per field, $n = 5$ (up to 100 cells per field). $^{*}P < 0.05$ versus control condition. (c) Representative Western blots from 3 independent experiments showing total protein levels of Cx43 and Panx1. Cell lysates were obtained from EOC20 cells under control conditions (lane C) or after the following treatments: TNF-α plus ATP (lane 1), IL-6/TNF-α plus ATP (lane 2), TNF-α/IFN-γ (lane 3), IL-6/TNF-α/IFN-γ (lane 4), TNF-α/IL-1β (lane 5), and IL-6/TNF-α/IL-1β (lane 6). Quantitation of Cx43 and Panx1 is shown; β-actin was used as a loading control for densitometric analysis.

4. Discussion

In this study, we demonstrated that extracellular ATP is required and advances the TNF-α/IFN-γ-induced dye coupling in cultured microglia, in an IL-1β-dependent manner. TNF-α/IFN-γ, but not TNF-α plus ATP enhances the basal and ATP-induced membrane permeability mediated by HCs. The increase in dye coupling induced by TNF-α/IFN-γ or TNF-α plus ATP was blocked by IL-6. Furthermore, inhibition of HCs prevents the pro-inflammatory molecules-induced upregulation of GJCs.

The ATP effects on the TNF-α/IFN-γ-induced dye coupling could be explained by activation of P2X receptors via ATP release, because the TNF-α/IFN-γ-induced dye coupling was prevented by oATP, a P2X receptor blocker. Activation of P2X receptors in microglia rises the $[Ca^{2+}]_i$ [1], which is known to induce gap junctional communication between cultured microglia in a PKC-dependent manner [24]. In agreement with the latter, BAPTA loaded microglia did not present dye coupling after treatment with TNF-α plus ATP. Thus, it is suggested that rises in $[Ca^{2+}]_i$ together with other downstream pathways contribute to up-regulate Cx43 levels and formation of HCs and GJCs as observed in other cell types [45, 67]. In HeLa cells expressing Cx43, rises in $[Ca^{2+}]_i$ enhance the cell surface levels of Cx43 HCs [45], a response that is directly associated to ATP release [68]. Thus, rises in $[Ca^{2+}]_i$ might contribute to increase the number of HCs in the plasma membrane of microglia. The increase in $[Ca^{2+}]_i$ could be initially mediated by activation of P2X receptors, but later on HCs might also contribute to increase their own activity favoring the Ca^{2+} influx because they are permeable to Ca^{2+} [69–71].

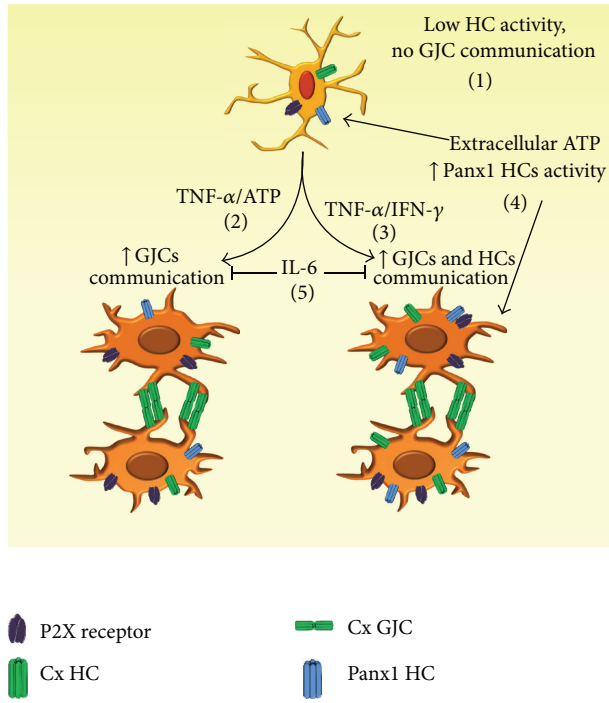

Figure 8: Cytokine-induced activation and the effect on gap junctional communication and HCs activity in cultured microglia. (1) Under resting condition, microglia express P2X receptors, Cx43, and Panx1, which have a low activity. Furthermore, no gap junction channel (GJC) communication is observed. (2) After TNF-α plus ATP exposition activated microglia exhibit gap junctional communication, but not intercellular communication mediated by hemichannels (HCs). (3) However, treatment with TNF-α/IFN-γ increased both GJC and HC functional state. (4) Extracellular ATP increases the Panx1 HC activity in both, resting or TNF-α/IFN-γ-activated microglia. (5) IL-1β release from activated microglia favors gap junctional communication. (6) IL-6 prevents IL-1β release and the increase in GJC and HC functional state.

The cytokine-dependent induction of gap junctional communication between microglial cells was transient, as previously observed in dendritic cells and monocytes/macrophages [50, 51, 72]. The transient response might be explained by the production and release of anti-inflammatory cytokines, such as IL-6, IL-10, and TGF-β, by activated microglia [1]. Accordingly, IL-6 drastically reduces the cytokine-induced dye coupling between microglia treated with TNF-α plus ATP or TNF-α/IFN-γ as it also occurs in dendritic cells treated with TNF-α/IL-1β [50]. Since IL-6 reduces cell adhesion in breast cancer cells [73], a similar mechanism might affect the stability of cellular contacts between microglia, impairing gap junctional communication. In addition, IL-6 was found to prevent the rise in $[Ca^{2+}]_i$. This might explain the inhibition of TNF-α plus ATP, because IL-6 did not prevent the increase in Panx1 levels. Although, IFN-γ signaling positively regulates purinergic receptors in microglia [11, 74], this might not explain the increase in dye coupling induced by TNF-α/IFN-γ because we found that IFN-γ delayed the appearance of dye coupling induced by TNF-α plus ATP. Further studies are required to unveil the mechanism underlying this cellular response.

We also found that in addition to TNF-α/IFN-γ, extracellular ATP and IL-1β also positively modulate the formation of GJCs in microglia. The link between purinergic signaling and IL-1β release has been well established in microglia [75], and here it was corroborated in EOC20 cells using IL-1ra, which prevented IL-1β release and establishment of dye coupling upon treatment with TNF-α plus ATP or TNF-α/IFN-γ. Interestingly, pro-inflammatory-like conditions (TNF-α/IL-1β or supernatant of microglia pretreated with LPS) increase HC activity but decrease gap junctional communication in primary astrocytes cultures [38]. However, we observed that TNF-α/IFN-γ increases both HC and GJC activity in microglia, indicating that different mechanisms control the functional expression of these channels in astrocytes and microglia.

As shown in this work, the activity of microglial Cx and Panx HCs was increased by TNF-α/IFN-γ. Interestingly, Panx1 HCs and several Cx HCs are pathways of ATP release to the extracellular space in several cell types including astrocytes and microglia [25, 35, 37, 76, 77]. Therefore, enhanced HC opening may control ATP release from activated microglia maintaining a higher $[Ca^{2+}]_i$ compared with resting microglia [78]. Extracellular ATP could open Panx1 HCs, which are also activated after TNF-α/IFN-γ, leading to release of IL-1β [31]. Because, the HC activity remains low after treatment with TNF-α plus ATP, even after acute application of ATP, we propose that under these conditions

ATP released by microglia via HCs was not required to induce IL-1β release. The latter is consistent with the prevention of TNF-α/IFN-γ-, but not TNF-α plus ATP-induced dye coupling in EOC20 cells treated with ^{10}Panx1, a Panx1 HC blocker. In addition, we speculate that after treatment with TNF-α plus ATP P2X receptors also contribute in a Panx1 HC-independent way, as it has been proposed to occur during microglial proliferation [79]. The role of Cx43 HCs in TNF-α/IFN-γ–induced dye coupling was confirmed using Cx43$^{(E2)}$ antibody, a specific Cx43 HC blocker. However, this conclusion should be taken cautiously because it was recently shown that several hours after Cx43$^{(E2)}$ antibody application, gap junctional communication is partially reduced [42].

Under control conditions microglial cells express low levels of Cxs [23, 24, 26–28]. Accordingly, in this study we detected low levels of Cx43 and also Panx1. However, brain damage or cytokine exposure promotes microglial activation, and under this condition they present elevated levels of Cx43 and become coupled through GJCs [23, 24, 27, 28]. Here we found that TNF-α in presence of IFN-γ upregulates Cx43 GJCs in microglia as it was previously demonstrated [23, 28]. In addition, and similar to dendritic cells [50], TNF-α/IL-1β increased Cx43 levels in microglia. On the other hand, IL-6 prevents the formation of GJCs induced by pro-inflammatory cytokines in dendritic cells [50]. Accordingly, we found that IL-6 efficiently prevented the pro-inflammatory molecules-induced increase in GJC and HC activity in microglia. This effect could be explained, at least in part, by prevention of Cx43 and Panx1 upregulation by IL-6 and prevention of IL-1β release.

So far, Panx1 has been demonstrated to form GJCs only in exogenous expression systems [71]. Together with the evidence that microglia from Cx43$^{del/del}$ mice do not express functional GJCs [23] and that Cx43$^{(E2)}$ antibody prevented the pro-inflammatory-induced dye coupling, it is suggested that dye coupling induced by TNF-α plus ATP or TNF-α/IFN-γ could be due to Cx43 GJCs. To recapitulate, we propose that in presence of extracellular ATP, Panx1 HC activity is enhanced and microglia migrate toward the injured site and release cytokines, as reported previously [33]. ATP could act in an autocrine and paracrine manner allowing IL-1β release and providing a pro-inflammatory microenvironment, which promotes an early up-regulation of Cx43 and Panx1, favoring the formation of HCs and GJCs in a stimulus-dependent manner (Figure 8). Later on, anti-inflammatory cytokines are produced and released to the extracellular milieu leading to reduction in intercellular communication mediated by HCs and GJCs similar to that of resting conditions. The latter is relevant because downregulation prevents a massive and/or prolonged ATP/glutamate release from microglia, which in turn can induce neurodegeneration [35, 56]. Thus, understanding the regulation of microglial purinergic receptors and intercellular communication via HCs and GJCs might contribute to modulate the timing of neuroinflammatory responses and led us to the identification of new therapeutic targets for neurodegenerative diseases [80].

Acknowledgments

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Review Article

Role of Neuroinflammation in Adult Neurogenesis and Alzheimer Disease: Therapeutic Approaches

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Neuroinflammation, a specialized immune response that takes place in the central nervous system, has been linked to neurodegenerative diseases, and specially, it has been considered as a hallmark of Alzheimer disease, the most common cause of dementia in the elderly nowadays. Furthermore, neuroinflammation has been demonstrated to affect important processes in the brain, such as the formation of new neurons, commonly known as adult neurogenesis. For this, many therapeutic approaches have been developed in order to avoid or mitigate the deleterious effects caused by the chronic activation of the immune response. Considering this, in this paper we revise the relationships between neuroinflammation, Alzheimer disease, and adult neurogenesis, as well as the current therapeutic approaches that have been developed in the field.

1. Introduction

The inflammatory response is an early, specialized immune reaction to tissue damage or pathogen invasion. In the central nervous system (CNS), this process is known as neuroinflammation and is characterized by the activation of the microglia and astrocytes population [1–3], the increase in concentration of different cytokines, and chemokines and, under certain conditions, the disruption of the blood brain barrier and the subsequent invasion of cells from the hematopoietic system to the injury site [4]. Thus, the burden of protecting CNS from injury falls on a specific group of cells: microglia, astrocytes, and mast cells. Mast cells can be found within the brain and their functions include the attractant and activation of other immune cells by secreting proinflammatory cytokines, and chemoattractants [5]. Astrocytes also contribute to the immune response by liberating both pro- and anti-inflammatory cytokines, chemokines and complement components [6]. Finally, microglia (CNS-resident macrophages) represent the main effector cells of the immune system in the CNS. Under physiological conditions, they stay as a quiescent population. In response to an infection or injury, they activate acquiring a reactive inflammatory phenotype characterized by an increased proliferation, morphological changes, and the release of several inflammatory molecules such as cytokines, reactive oxygen species, and nitric oxide [7].

Some aspects of the neuroinflammatory response result beneficial for CNS outcomes. Among these benefits, neuroprotection phenomena, the maintenance of neurogenesis as a mechanism of brain repair, the mobilization of neural precursors for repair, remyelination, and even axonal regeneration are included [8, 9]. However, neuroinflammation can be harmful too, leading to neuronal damage. Benefits and detriments balance depends largely on the magnitude of the immune response. In this sense, it is important to distinguish between the two types of responses in which inflammatory mechanism has traditionally been classified: acute and chronic inflammation. The first one comprises the immediate and early response to an injurious agent and is basically a defensive response that paves the way for repair of the damaged site being typically short-lived and unlikely to be detrimental to long-term neuronal survival [10]. The chronic

response occurs when the harmful stimulus persists over time and contrary to the acute form, it is a long-standing and often self-perpetuating neuroinflammatory response which in the end, results in detrimental consequences for neurons [11]. Both acute and chronic responses have been related with neurodegenerative disorders. Thus, stroke and injury would associate with acute neuroinflammation while diseases such as multiple sclerosis or Alzheimer disease (AD) would be associated with the chronic form of the response. In this scenario, another important process commonly related with neurodegeneration and neuroinflammation emerges; the formation of new neurons in the adulthood or adult neurogenesis.

Adult neurogenesis occurs in mammals principally in two brain regions: the subventricular zone (SVZ) and the subgranular zone (SGZ) of the hippocampus. The neuronal precursor cells (NPCs) that exist in both areas are a subset of astrocytes that give rise to intermediate progenitors which migrate and differentiate into new neurons of the hippocampus (in SGZ neurogenesis) or the olfactory bulb (in SVZ neurogenesis) [12]. Adult neurogenesis has been found to be altered in several neurodegenerative disorders such as Parkinson's disease, Huntington's disease, and AD (for a review, see [13]). On the other hand, neuroinflammation is a common feature of all these pathologies and, as it will be commented in detail later, it has also a relevant influence on adult neurogenesis.

Due to the interconnection among these processes, it is important to consider them as a whole, taking into account that alterations affecting any of them would probably have consequences on the two others.

In this review, we will summarize the role of neuroinflammation in both adult neurogenesis and Alzheimer disease. Related to this pathology, we will finally revise the recent advances concerning therapeutic approaches with neuroinflammatory mechanisms as a main target.

2. Neuroinflammation and Adult Neurogenesis

It has been widely demonstrated that neuroinflammation affects adult neurogenesis having both detrimental and beneficial consequences which can result in enhancement and/or inhibition of the process. The final result depends largely on how microglia, macrophages, and/or astrocytes are activated and the duration of the inflammation [14]. Furthermore, the balance between the benefits and the detriments will have a profound impact on the efficiency of brain repair [15], which is of great importance in the context of the neurodegenerative disorders.

It is known that microglia, as the first immunological barrier against pathogens and environmental insults [16], exert the aforementioned dual effects on adult neurogenesis, resulting in pro or antineutogenic outcomes.

2.1. Proneurogenic Effects. An important study performed by Sierra and colleagues demonstrated that resting microglia play a crucial role in regulating the balance of newborn neurons in the hippocampus thanks to their phagocytic capacities [17]. Of the thousands of new cells born in the SGZ of the dentate gyrus, only a part of them differentiate and maturate into fully mature neurons. At least half of these cells die, probably through apoptosis, within the first few days to weeks after they are born [18–21]. Sierra et al. [17] provided data that attributed to microglia the function of removing those apoptotic new cells by phagocytosis. Furthermore, they importantly proved that this action did not require the activation of the microglial population. Recently, it has been reported that not only microglia are essential for adult neurogenesis but their functions and activity are importantly regulated by neuronal progenitor cells too. Thus, NPC-derived secreted factors are capable of modulating microglia activation, proliferation, and phagocytosis [22]. This crosstalk persists during neuron lifetime, since adult neurons are demonstrated to regulate microglia activation by constitutively expression of several neuroimmunoregulatory proteins such as CD200, CX_3CL1 (or fractalkine), CD47, CD55, or HMGB1 (for a review see [23]).

Further evidence of the proneurogenic effects of unchallenged microglia comes from the work of Walton and colleagues. By *in vitro* studies they showed that this population releases factors that rescue neuroblasts and instruct neuronal cell differentiation [24].

However, not only resting microglia exert benefits on adult neurogenesis. The acquisition of an active phenotype, under certain conditions, can be beneficial too [16], thanks to the liberation of anti-inflammatory cytokines with a broad range of actions on neurogenesis. Among these, we can highlight interleukin-4 and -10 (IL-4, IL-10) and transforming growth factor-beta (TGF-β). It is also important to keep in mind that cytokines classically considered as proinflammatory, such as interleukin-6 (IL-6), interleukin 1-β (IL-1β), and tumor necrosis factor-α (TNF-α) can be involved in the creation of a permissive environment for neurorepair too [25], as several studies have demonstrated (see Table 1).

Finally, not only cytokines derived from microglia can positively regulate neurogenesis. Other factors produced by immune system cells which are involved in the neuroinflammatory response have been shown to have certain influence. This is the case for granulocyte-macrophage colony stimulating factor (GM-CSF) [26] and the granulocyte-colony stimulating factor (G-CSF), with remarkable effects on the differentiation of NSC *in vitro* [27] (Table 1).

2.2. Antineurogenic Effects. Neuroinflammation, although beneficial as a physiological response to maintain brain homeostasis, can have detrimental effects especially when it turns out to be a chronic response. Activated miroglia release proinflammatory cytokines which have been shown to affect largely neurogenesis. The aforementioned IL-6, TNF-α, and IL-1β but also interleukin-1-alpha (IL-1α), interleukin-18 (IL-18) and interferon-γ (IFN-γ) have detrimental consequences for proliferation and/or differentiation of NSC (Table 1). Among factors not released by microglia, CCL11 or eotaxin-1, a small chemokine known by its implication in allergic responses, has been recently linked to adult neurogenesis and ageing [28]. When administered systemically to young mice,

eotaxin is able to impair neurogenesis producing as a consequence, learning and memory deficits [28]. Furthermore, it seems to affect directly the number and size of neurospheres formed from primary NPCs [28], suggesting that precursor cells probably have receptors capable of binding the cytokine.

Finally, it is especially noteworthy to keep in mind that most of the aforementioned factors are not only produced by microglia, but for astrocytes too, which contribute to the pathogenesis of neurodegenerative disorders as will be commented later.

The effects of the abovementioned cytokines on neurogenesis are summarized in Table 1.

3. Neuroinflammation and Alzheimer Disease

Neurodegenerative diseases are characterized by the progressive loss of neurons from specific regions of the CNS, which is believed to account for the cognitive and motor impairments suffered by patients with these neurodegenerative disorders. Importantly, inflammation is a process that has been closely related to the onset of many of these diseases, such as Amyotrophic Lateral Syndrome (ALS), Multiple Sclerosis (MS), Parkinsons Disease (PD) and Alzheimer Disease (AD) [29–33]. Indeed, aberrant inflammatory responses are believed to play a role in the etiology of these disorders.

At present, AD is the most common cause of dementia in the elderly. It is estimated that 27 million people are affected worldwide [34] and this number is expected to triple by 2050 due to the increase of the population life expectancy [35]. AD is a neurodegenerative disorder which affects brain regions that control memory and cognitive functions, which implies that patients finally lose their memory and ability to learn, to reason, to communicate and to carry out daily activities [36]. There are two different types of Alzheimer Disease, familiar Alzheimer Disease (FAD) and Sporadic Alzheimer Disease (SAD), and the origin of the disease could be different in both familial and sporadic cases. In terms of FAD, mutations in three different genes (presenilin-1 PS-1, presenilin 2 PS-2 and amyloid precursor protein APP) are likely to promote the onset of the disease whereas for SAD, different risk factors might be involved. Nevertheless, downstream the initial causes of the disease some common factors may be involved [37]. At a molecular level, AD is characterized by the presence of two main hystopathological hallmarks: senile plaques (extracellular aggregates composed by amyloid peptide or Aβ) and neurofibrillary tangles (intracellular aggregates composed by hyperphosphorylated forms of tau protein). Aβ results from the cleavage of APP and, although it seems to have important developmental functions in cell differentiation and possibly in the establishment of synapses [38, 39], its functions in adult brain still remain unclear. On the other hand, tau protein, the major component of neurofibrillary tangles, is a microtubule associated protein which contributes to the normal function of this intracellular support structure. Under pathogenic conditions, tau is highly phosphorylated reducing its ability to bind to microtubules [40] and favoring the formation of protein aggregates.

As a part of the inflammatory response, gliosis is a common feature of AD. Activated astrocytes and microglia are characteristically found in abundance near neurons and plaques. Besides, AD brains show increased expression of several pro-inflammatory cytokines which are hardly found in normal brains [61–64]. The main hypothesis proposes the chronic inflammatory reaction as a response to the accumulation of Aβ plaques and tangles [65]. Although initial inflammatory response can be beneficial, chronic activation of astrocytes and microglia has been shown to induce necrosis in adjacent neurons by releasing reactive oxygen intermediates, nitric oxide, proteolytic enzymes, complementary factors, or excitatory amino acids [66].

Aβ and their precursor APP are potent activators of glial cells [67, 68]. Thus, Aβ binds to the microglial cell surface regulating extracellular signal regulated kinase (ERK) and mitogen-activated protein kinase (MAPK) pathways which induces proinflammatory gene expression leading to cytokine and chemokine production [69]. Several chemokines and their receptors have been found to be upregulated in the AD brain. For example, macrophage inflammatory protein (MIP)-1α has been detected in reactive astrocytes nearby Aβ plaques [70]. In the same manner, changes in levels of many cytokines have been described not only in AD brains but also in blood and cerebrospinal fluid from patients. Thus, increased levels of IL-1α, IL-1β, IL-6, TNF-α, and GM-SF have been reported in brain tissue [71, 72]. In serum from patients, an increase in eotaxin, a cytokine recently linked to adult neurogenesis and ageing has been also detected [28] and, correlating to this, an increase in the expression of its receptor, CCR3, has been found in AD brains, especially in microglia [73]. Importantly, several works describe interactions between components of the senile plaques and cytokines, which could be generating a positive feedback loop for the neuroinflammatory process [74]. For example, Aβ protein is able to potentiate the secretion of IL-6 and IL-8 under several conditions [75]. Similarly, astrocytes might be activated by Aβ [76], contributing to generating a proinflammatory environment via the liberation of several cytokines and chemokines.

However, in some situations the role of microglia has been shown to be beneficial, since the activation of this population can decrease the accumulation of Aβ thanks to their phagocytic ability which facilitates the clearance and degradation of the aggregates [77]. Besides, microglia can be beneficial too through the secretion of growth factors such as the glia-derived neurotrophic factor (GDNF) which favors neuron survival [78]. Similarly, a relatively unknown cytokine, fractalkine, which has been demonstrated to have important neuroprotective characteristics, has been recently linked to the disease. Thus, fractalkine signaling (with its only receptor CX_3CR1) has been found to be altered in AD brains in which reduced levels of the cytokine has been described [79].

Finally, it is noteworthy to keep in mind that although neurons have been traditionally believed to be passive bystanders in neuroinflammation, they seem to contribute to the production of neuroinflammatory molecules, a phenomenon that could be relevant in AD. Thus, the

Table 1: Effects of different cytokines on neurogenesis.

Cytokine	Effects on neurogenesis	References
IL-1α	Increased astrocyte lineage	[41]
IL-1β	Stimulation of NPCs proliferation and differentiation	[42]
	Decreased proliferation, survival, and neuronal differentiation	[43]
	Increased astrocyte differentiation	[44]
IL-4	Increased oligodendrogenesis	[45]
IL-6	Decreased proliferation, survival, and neuronal differentiation	[46]
	Differentiation of NSC to neuronal lineages	[47]
	Increased neurogenesis	[48]
		[49]
IL-10	Increased proliferation	[50]
		[51]
IL-18	Decreased survival	[52]
	Increased neuronal differentiation	
IFN-γ	Decreased proliferation and survival of multipotent progenitors	[53]
	Promotion of differentiation and neurite outgrowth	[54]
		[55]
CCL11 (eotaxin-1)	Decreased Sox-2 progenitors, proliferation, and neuronal differentiation	[28]
CX3CL1 (fractalkine)	Decreased neurogenesis	[56]
GM-CSF	Stimulation of NPCs differentiation	[57]
G-CSF	Promotes NPCs differentiation	[27]
TGF-β	Decreased proliferation	[58]
	Increased survival and neural differentiation	[59]
		[60]

production of IL-1, IL-6, and TNF-α by neurons has been reported. Indeed, these neuronal chemokines act as messengers between neurons and glial cells (for a review, see [80]).

As neuroinflammation represents an important hallmark in AD and, as it has been shown in Section 2, it has a remarkable influence on adult neurogenesis, modulating the inflammatory environment could be beneficial not only for improving the deficits directly provoked by the disease but also for stimulating the endogenous ability of the brain for repairing the damage. In this sense, it is important to highlight that, especially in AD, understanding the role of adult neurogenesis is of great importance considering that one of the neurogenic zones is the hippocampus, structure responsible for cognitive and learning capacities which is largely affected in AD patients.

To date, it is not fully understood how adult neurogenesis is affected in neurodegenerative disorders. In AD, contradictory results have been obtained from the study of several animal models and the study of brain tissue by biochemical and histological approaches. Different effects on proliferation, differentiation, and survival have been reported in AD transgenic animal models with mutations in APP and tau or in both (for a review, see [81]). Besides, alterations affecting NPCs and differentiation of newborn neurons have been described in a glycogen-synthase kinase 3 overexpressing mouse model (GSK-3β has been proposed as a key protein in AD [82]), with an important role of microglia as a mediator of these damaging effects [83, 84] among which, morphology alterations of newborn neurons are included [85]. In humans, first data were obtained by Nagy and colleagues in 1997 [86]. In this pioneer work, the authors reported an increase in Ki-67 marker (staining proliferating cells) in the hippocampus from AD patients. In 2004, Jin et al. confirmed this result restricting it specifically to neurons [87]. However, in 2006, another group, although reported an increase in the proliferative status of presenile AD brains, they demonstrated that these precursors finally differentiated into glial cells [88].

Consequently, although adult neurogenesis remains an unknown field to be further explored in Alzheimer disease, it is likely to be affected in the disease. Taking into account that this process is known to contribute to learning and memory [89–91], an appropriate form to improve the subsequent deficits in cognitive functions associated to AD would result from modulating factors, such as those implicated in neuroinflammation, directly related to the correct formation of the newborn neurons. Finally, we cannot forget that adult neurogenesis declines with age, being a not so common event in the elderly [92], a fact that reinforces even more the idea of preserving or stimulating it as a brain repair mechanism.

4. Therapeutic Approaches

Based on the evidence that involves neuroinflammation in the pathogenesis of Alzheimer disease, researchers have focused their efforts on the development of anti-inflammatory drugs as a treatment option for patients with AD. Drugs such as the NSAIDs and glucocorticoid steroids have been studied.

4.1. NSAIDs. NSAIDs is the abbreviation for "nonsteroidal anti-inflammatory drugs." They constitute a large family of

compounds which includes the salicylate, propionic acid, acetic acid, fenamate, oxicam, and the COX-2 inhibitor classes (enzymes which regulate the homeostatic production of prostanoids, implicated in the inflammatory response) [36]. Epidemiological evidences indicate that NSAIDs may lower the risk of developing AD [93–95], since patients suffering from rheumatoid arthritis and osteoarthritis have been shown to inversely correlate with the risk of develop AD. Although beneficial effects have been observed both *in vitro* and *in vivo* (for a review, see [80]), unfortunately, clinical trials of NSAIDs in AD patients have not been very fruitful [96], especially in the case of COX-2 inhibitors. Thus, COX-2 inhibitor rofecoxib and the COX-1 and COX-2 inhibitor naproxen, were unable to slow the progression of the disease in patients with mild-moderate AD [97]. As a possible hypothesis, it could be postulated that NSAIDs might be useful to prevent the pathology but ineffective once the disease occurs.

4.2. Glucocorticoid Steroids. These compounds are considered as potent anti-inflammatory agents that modulate the transcription of several inflammatory molecules reducing, for example, the expression of proinflammatory cytokines and complement proteins [98]. However, the results obtained in AD patients have not been very promising. Thus, the use of some glucocorticoid steroids, such as prednisone, has not revealed any benefit in terms of slowing cognitive decline [99].

However, other therapies have been developed not directly directed to reduce inflammation but to the main targets that induce the chronic activation of these mechanisms, such as Aβ plaques or tau protein.

4.3. Aβ-Based Immunization Strategies. the efficacy of these therapies has been demonstrated in mouse models of the disease. In 1999, Schenk and colleagues proved in an APP mutant mice that Aβ-directed vaccination prevented the development of neuritic Aβ plaques reducing them in older animals [100]. Furthermore, vaccination was effective in reducing age-dependent learning deficits which correlated with reductions in both soluble Aβ and tau [101]. Although APP model does not recapitulate all common features of AD, they resemble an early preclinical phase of the disease, which may be the optimal phase to initiate a therapy for preventing the disorder [102]. Importantly, efficacy of the vaccine was also found in a nonhuman primate, the Caribbean vervet [103]. Regarding the promising results obtained in animal models, a clinical trial was launched with AN-1792 containing preaggregated synthetic Aβ_{42} and the adjuvant QS-21 [104]. Although 6% of the patients developed meningoencephalitis, some others developed Aβ-antibody titres that correlated with a slow cognitive decline [105], and this result encouraged the development of several antibody fragments and humanized Aβ-specific antibodies, which are currently in various stages of clinical trials [102]. Time will tell whether these therapies are effective enough to halt the disease.

4.4. Tau-Based Immunization Approaches. First approach applying tau-based immunization was carried out by Rosenmann and colleagues in 2006 by injecting C57BL/6 wild-type animals with full-recombinant human tau. The experiments are unsuccessful since the vaccination caused encephalitis [106]. Subsequently, other groups tried active immunization approaches using tau phosphopeptides, obtaining promising results in tau transgenic models, in which they were able to prevent tau pathology in the absence of obvious side effects (for a review, see [107]). However, one of the main problems derived from these studies is the difficulty to translate them into clinical practice. This is due to the fact that the vaccinations were observed to prevent tau-related problems when administered prior to the appearance of any pathology or cognitive deficit, something that, nowadays, would be impossible regarding the current diagnosis methods. At present, the tau-targeted therapies that are in clinical trials target tau phosphorylation by GSK-3, microtubule stability, and aggregation [108].

Finally, it is important to highlight that considering that Aβ pathology depends on the presence of tau [109, 110] and that Aβ deposition is absent in many tauopathies (neurodegenerative diseases associated with the pathological aggregation of tau), it is absolutely necessary to pursue a tau-targeted treatment probably in combination with an Aβ-targeting approach.

5. Concluding Remarks

Although mechanisms underlaying Alzheimer disease remain unclear, neuroinflammation seems to be a common feature to neurodegenerative diseases with an important contribution to the pathology, affecting among others, physiological processes with a repairing function such as the adult neurogenesis process. Thus, modulating neuroinflammation by targeting causing agents or/and trying to ameliorate their harmful effects could be of great importance to possibly, prevent AD pathology and contribute to stimulate endogenous repairing mechanisms as the formation of new neurons.

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