



NEUROIMMUNE INTERFACE IN HEALTH AND DISEASES

EDITED BY: Ihssane Zouikr, Sanae Hasegawa-Ishii and
Atsuyoshi Shimada

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NEUROIMMUNE INTERFACE IN HEALTH AND DISEASES

Topic Editors:

Ihssane Zouikr, RIKEN BSI, Japan

Sanae Hasegawa-Ishii, Kyorin University, Japan

Atsuyoshi Shimada, Kyorin University, Japan

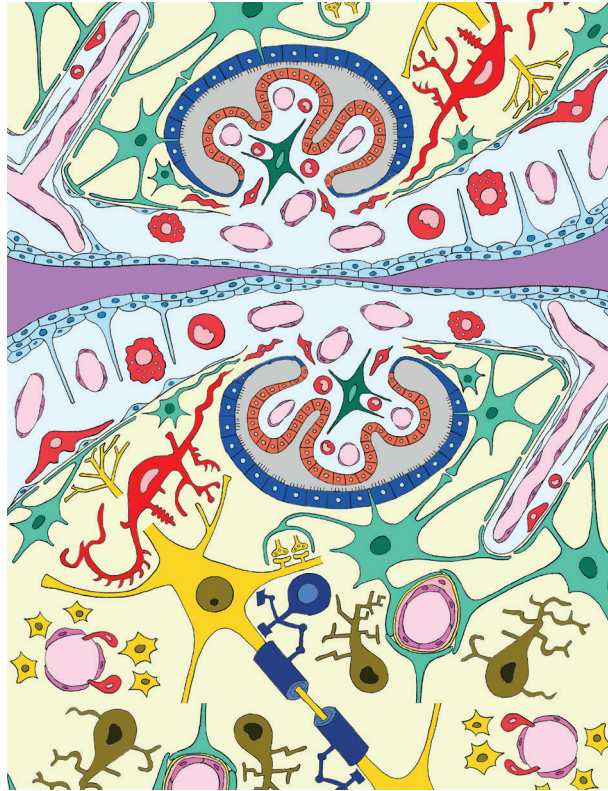
It is now well appreciated that the immune system, in addition to its traditional role in defending the organism against pathogens, communicate in a well-organized fashion with the brain to maintain homeostasis and regulate a set of neural functions. Perturbation in this brain-immune interactions due to inflammatory responses may lead to psychiatric and neurological disorders.

Microglia are one of the essential cells involved in the brain-immune interactions. Microglial cells are now not simply regarded as resident tissue macrophages in the brain. These cells are derived from myeloid progenitor cells in the yolk sac in early gestation, travel to the brain parenchyma and interact actively with neurons during the critical period of neurogenesis. Microglia provide a trophic support to developing neurons and take part in the neural wiring through the activity-dependent synapse elimination via direct neuron-microglia interactions.

Altered microglial functions including changes in the gene expression due to early life inflammatory events or psychological and environmental stressors can be causally related to neurodevelopmental diseases and mental health disorders. This type of alterations in the neural functions can occur in the absence of infiltration of inflammatory cells in the brain parenchyma or leptomeninges. In this sense, the pathogenetic state underlying a significant part of psychiatric and neurological diseases may be similar to “para-inflammation”, an intermediate state between homeostatic and classical inflammatory states as defined by Ruslan Medzhitov (Nature 454:428-35, 2008). Therefore, it is important to study how systemic inflammation affects brain health and how local peripheral inflammation induces changes in the brain microenvironment.

Chronic pain is also induced by disturbance in otherwise well-organized multisystem interplay comprising of reciprocal neural, endocrine and immune interactions. Especially, early-life insults including exposure to immune challenges can alter the neuroanatomical components of nociception, which induces altered pain response later in life.

Recently the discrete roles of microglia and blood monocyte-derived macrophages are being defined. The distinction may be further highlighted by disorders in which the brain parenchymal tissue is damaged. Therefore, studies investigating the dynamics of immune cells in traumatic



Schematic illustration of the sites of brain-immune cell-cell interaction.

Red-colored cells represent bone marrow-derived myeloid cells that are located in the subarachnoid space, perivascular space and choroid plexus stroma, all of which are contiguous (indicated in pale blue). Bone marrow-derived cells enter the subarachnoid space through fenestrated blood vessels and can be localized in close apposition to the pial and subpial astrocytic endfeet that are a part of the brain parenchyma.

Marrow-derived cells in the perivascular space can be localized in close apposition to perivascular astrocytic endfeet. Marrow-derived cells can cross the loose blood vessel walls of the circumventricular organs and enter the brain parenchyma (indicated in pale yellow). Marrow-derived cells in the choroid plexus stroma can migrate into the brain parenchyma through the attachments of choroid plexus without being blocked by the blood brain barrier. These cells dramatically change their shape into a ramified morphology once they enter the parenchyma. Therefore, immune cells can interact with brain parenchymal cells via at least four types of histological architecture: subarachnoid space, perivascular space, circumventricular organs and attachments of choroid plexus.

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brain injury and neurotropic viral infections including human immunodeficiency virus, etc. as well as neurodegenerative diseases such as amyotrophic lateral sclerosis are promising to clarify the interplay between the central nervous and immune systems. The understanding of the histological architecture providing the infrastructure of such neuro-immune interplay is also essential.

This Frontiers research topic brings together fourteen articles and aims to create a platform for researchers in the field of psychoneuroimmunology to share the recent theories, hypotheses and future perspectives regarding open questions on the mechanisms of cell-cell interactions

with chemical mediators among the nervous, immune and endocrine systems. We hope that this platform would reveal the relevance of the studies on multisystem interactions to enhance the understanding of the mechanisms underlying a wide variety of neurological and psychiatric disorders.

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Editorial: Neuroimmune Interface in Health and Diseases

Ihssane Zouikr^{1*}, Sanae Hasegawa-Ishii² and Atsuyoshi Shimada³

¹Laboratory for Molecular Mechanisms of Thalamus Development, RIKEN BSI, Wako, Japan, ²Department of Pharmacology, Pennsylvania State University College of Medicine, Hershey, PA, United States, ³Faculty of Health Sciences, Kyorin University, Tokyo, Japan

Keywords: neuron–glia interactions, amyotrophic lateral sclerosis, pain, BBB transport, mood disorders, neuroinflammation, HIV cure research, bone marrow-derived cells

Editorial on the Research Topic

Neuroimmune Interface in Health and Diseases

Neuroimmunology is a field that investigates the bi-directional communication between the nervous system (CNS and PNS) and the immune system. While these two physiological systems were traditionally thought to act independently and that the brain was a privileged site protected by the blood–brain barrier (BBB), researchers now appreciate the highly organized cross talk between the immune and nervous systems in health and disease. This conceptual shift came with a series of pioneering experiments by Hugo Besedovsky who demonstrated that the hypothalamic–pituitary–adrenal (HPA) axis and the sympathetic nervous systems were activated in response to a foreign antigen in rats (1). Injection of sheep red blood cells in rats coincides with enhanced levels of circulating corticosterone as well as decreased noradrenaline turn over in the hypothalamus of rats. With Charles Dinarello, who first cloned interleukin (IL)-1 β , Besedovsky demonstrated that IL-1 β is responsible for the development of the brain response to peripheral immune stimulation (2, 3). During the same year, Ed Blalock demonstrated that the production of adrenocorticotrophic hormone by leukocytes is induced by corticotropin-releasing hormone (3, 4). The CNS communicates with the immune system *via* hormonal and neural pathways. The hormonal pathway is predominantly *via* the HPA axis, which is the primary stress center in rodents, primates, and humans. The neural pathway is mediated *via* the sympathetic and parasympathetic (the vagus nerve) response. In turn, the immune system signals the CNS *via* cytokines released by activated immune cells in the periphery but also through activated microglia and astrocytes in the spinal cord and brain. The peripheral inflammation can lead to central proinflammatory milieu and ultimately to sickness behavior defined as a set of behavioral changes that develop in individuals during the course of systemic inflammation (i.e., fever, lethargy, hyperalgesia). Cytokines released at the periphery can reach the brain through the circumventricular organs, areas devoid of BBB such as the organum vasculosum lamina terminalis, or through transport and secretion by BBB cells (5).

The access of immune cells and other mediators to the CNS is controlled by the BBB. However, in the event for instance of brain or spinal cord trauma, exposure to environmental toxicants, and inflammation including infection and autoimmune diseases, etc. this barrier can be breached. BBB dysfunction may be seen in a number of neurodegenerative disorders (6) including amyotrophic lateral sclerosis (ALS). Patients with ALS were shown to have enhanced neurovascular permeability (7). However, whether BBB breakdown is the cause or consequence of ALS is still unknown and more studies are needed to clarify the role of BBB breakage in the onset of ALS and of other neurodegenerative disorders.

McKee and Lukens summarize in their review paper the current understanding of the role of immune cells in traumatic brain injury pathogenesis (McKee and Lukens), while Imamura and

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Robert Weissert,
University of Regensburg, Germany

*Correspondence:

Ihssane Zouikr
ihssane.zouikr@riken.jp

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Hasegawa-Ishii discuss the immune response in the olfactory mucosa following exposure to environmental toxicants (Imamura and Hasegawa-Ishii).

Pain, is the topic of four articles in this special issue. Zouikr and Karshikoff provide an in-depth overview of the role of the cross talk between the endocrine, immune, and central nervous systems in pain as well as the importance of taking early life history into account when treating patients with chronic pain (Zouikr and Karshikoff). Barr and colleagues demonstrate that nerve and root compression in postnatal (P) day 10, 14, 21, and 28 rats produced thermal hyperalgesia and mechanical allodynia that was accompanied by enhanced proinflammatory cytokines and chemokines in the spinal cord. The extent of hyperalgesia and immune activation was greater in older animals (Barr et al.). Pain is a common symptom among patients with multiple sclerosis (MS), using an animal model of MS-induced pain, namely, experimental autoimmune encephalomyelitis (EAE), the team lead by Moalem-Taylor showed that even prior to the onset of clinical EAE, mice already developed mechanical allodynia. This coincided with enhanced levels of Iba1, a marker of microglia, in the spinal cord. Mice with EAE also exhibited increased facial grimacing in the mouse grimace scale during clinical disease (Duffy et al.). Hua in her review argues that opioid-containing immune cells play an important role in peripheral analgesia in inflamed tissue.

Disruption of immune to brain communication is known to increase the susceptibility of developing psychopathological and neurological disorders. In his review, Neupane argues that dysfunctioning of the neuroimmune system could influence the development, progression, and outcome of alcohol use disorder (AUD) and major depression (MD) comorbidity and therefore neuroimmunological alteration should be taken as a key pathophysiological factor when considering the comorbidity between AUD and MD. Karshikoff and Lasselin review the current understanding on the relationship between inflammation and fatigue and argue that the multidimensional aspect of fatigue should be considered when investigating inflammation-induced fatigue (Karshikoff et al.). Edmonson et al. provide a very informative review on the important role of microglia in maintaining a healthy neural network and that abnormal microglial activity can lead to autism. Of particular importance, abnormalities of microglia at the genetic and epigenetic level may contribute to the pathogenesis of autism spectrum disorder. Over-reactivity of microglia has been identified in patients with bipolar disorder, Ohgidani et al. developed a genius technique to induce microglia-like from monocytes. They found a downregulation of CD206, a mannose receptor expressed at the surface of macrophages, endothelial cells, and dendritic cells, during the manic state among three patients with bipolar disorder. This is an important translational study that provides the first evidence that the gene

profiling patterns are different between manic and depressive states (Ohgidani et al.). Maintaining a healthy microglia–neuron interaction is critical for neuronal function homeostasis, Wohleb provides an in-depth overview of the importance of neuron–microglia communication in determining homeostatic neuronal function and that alteration in this bi-directional communication can lead to mental health disorders. Microglia can constitute a reservoir for the human immunodeficiency virus (HIV) that use these immune cells to silence its transcription producing a state of viral latency. Marban et al. discuss the different molecular mechanisms involved in the establishment and persistence of HIV latency in brain reservoirs as well as the importance of understanding these molecular mechanisms in order to purge or at least reduce the pool of latently infected brain cells.

The leptomeninges, choroid plexus, attachment of choroid plexus, perivascular space, circumventricular organs, and astrocytic endfeet construct the histological architecture that provides a location for intercellular interactions between bone marrow-derived myeloid lineage cells and brain parenchymal cells under non-inflammatory state but also during the early stages of systemic inflammation. Shimada and Hasegawa-Ishii propose a mechanism connecting systemic inflammation, brain-immune interface cells, and brain parenchymal cells and discuss the relevance of this immune to brain interaction in the context of neurological disorders.

Amyotrophic lateral sclerosis is a debilitating neurodegenerative disorder characterized by a progressive degeneration of motoneurons in the spinal cord and motor cortex. Bone marrow transplantation (BMT) is a promising approach to recompensate the loss of spinal motoneuron in ALS; however, adequate conditioning of BMT is a complex task. In their research article, Peake and colleagues showed that conditioning mice with higher dose of busulfan followed by BMT lead to higher accumulation of bone marrow-derived cells in the spinal cord which was due in part to proliferation of these cells, as well as enhanced microglial activity 7 weeks post-transplant. The authors also demonstrate that in mSOD mice (a mouse model of ALS) conditioned with busulfan, a much higher level of BMDCs accumulation in the spinal cord was observed compared to that of wild-type mice (Peake et al.). However, whether these transplanted stem cells differentiate into motoneurons and whether this is associated with functional recovery in ALS patients is still not known.

As editors, we would like to express our gratitude to all of the scientists around the globe who contributed to this special issue and we hope that you will enjoy reading it.

AUTHOR CONTRIBUTIONS

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A Non-inflammatory Role for Microglia in Autism Spectrum Disorders

Catherine A. Edmonson^{1,2†}, Mark N. Ziats^{2,3†} and Owen M. Rennert^{2*}

¹ University of Florida College of Medicine, Gainesville, FL, USA, ² National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD, USA, ³ Medical Scientist Training Program, Baylor College of Medicine, Houston, TX, USA

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Edited by:

Ihssane Zoulikr,
RIKEN Brain Science Institute, Japan

Reviewed by:

Andrew MacLean,
Tulane University School of
Medicine, USA
Anna Fogdell-Hahn,
Karolinska Institutet, Sweden

*Correspondence:

Owen M. Rennert
rennerto@mail.nih.gov

[†]Catherine A. Edmonson and
Mark N. Ziats contributed
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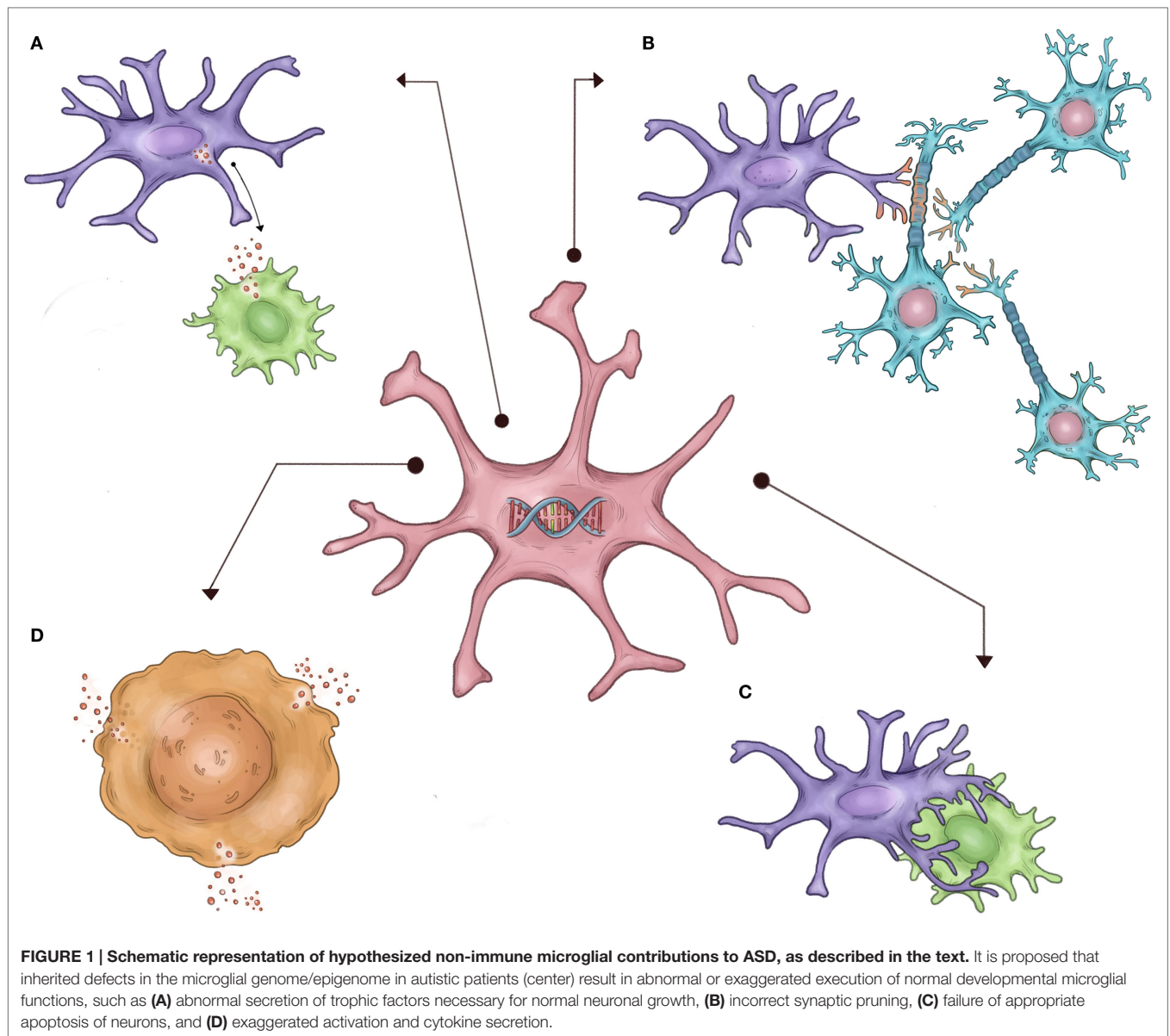
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Autism spectrum disorder (ASD) is a neurodevelopmental disorder characterized by deficits in social interaction, difficulties with language, and repetitive/restricted behaviors. The etiology of ASD is still largely unclear, but immune dysfunction and abnormalities in synaptogenesis have repeatedly been implicated as contributing to the disease phenotype. However, an understanding of how and if these two processes are related has not firmly been established. As non-inflammatory roles of microglia become increasingly recognized as critical to normal neurodevelopment, it is important to consider how dysfunction in these processes might explain the seemingly disparate findings of immune dysfunction and aberrant synaptogenesis seen in ASD. In this review, we highlight research demonstrating the importance of microglia to the development of normal neural networks, review recent studies demonstrating abnormal microglia in autism, and discuss how the relationship between these processes may contribute to the development of autism and other neurodevelopmental disorders at the cellular level.

Keywords: autism spectrum disorder, neurodevelopment, microglia, glia, neurodevelopmental disorders

INTRODUCTION

Autism spectrum disorder (ASD) is a clinical neurodevelopmental syndrome characterized by abnormalities in social interaction and language, and restrictive/repetitive patterns of interest (1). Despite the large and seemingly increasing prevalence of ASD (2), the biological mechanisms underlying the broad ASD phenotype remain unclear. However, strong evidence has recently implicated both immune pathways and independently, abnormalities in neural synaptogenesis, as potentially underlying the clinical ASD phenotype. However, it is unclear how these two seemingly distinct processes are related to the risk for the development of autism. A large body of research has suggested a link between inflammation in the CNS and resultant destruction of neural networks as one potential link between these two processes (3). However, microglia – the resident immune cells of the CNS – are increasingly implicated in *normal* neurogenesis and the formation of neural networks in the unaffected developing brain (4–6). Furthermore, microglia have also been repeatedly shown to be abnormal in postmortem autistic brain tissue and cellular/animal models of ASD (7–10). Therefore, it is equally likely that inherent abnormalities in microglia that are required to properly shape developing neural networks may link these two seemingly disparate processes separate from microglia's traditional role as immune cells (**Figure 1**). In this review, we highlight research demonstrating the importance of microglia to the development of normal neural networks, review recent studies demonstrating abnormal immune signaling and microglia in ASD, and discuss how the relationship



between these two emerging areas of neurodevelopment research may at least partially contribute to the development of ASDs at the cellular level.

DISCUSSION

Microglia in Normal Neurodevelopment

The microglial cells of the CNS derive from myeloid progenitor cells in the yolk sac in early gestation, and travel to the brain to establish residency by early embryonic development (11). Traditionally, cellular neuroscience research has considered glial cells as predominately supportive to neurons during normal neurogenesis. In particular, microglia were traditionally viewed very narrowly as only becoming activated in response to pathological insults, similar to their peripheral tissue counterparts, and thus contributing little to normal neurodevelopmental processes

(12–14). However, *in vivo* imaging techniques have repeatedly demonstrated that microglia are highly active during normal brain development and that they interact directly with neurons during critical period of neurogenesis (4, 5). Moreover, activity-dependent synapse and dendritic spine remodeling have been shown to be modulated in part by microglia in the mouse motor cortex and visual cortex (6). These and similar findings have helped to shift the view of normal cellular neurodevelopment from a primarily neuroncentric process to one that acknowledges the interactions between neurons and glia in properly wiring the brain's circuitry under normal physiological conditions.

Recent studies support the idea that microglial cells play a central role in early neurodevelopment; however, the specific mechanisms of how microglia contribute and what effect abnormalities in their contribution may have is only beginning to be understood. Some have suggested that microglia may be responsible

for releasing trophic factors essential for early neuronal growth. This idea is supported by studies demonstrating that cultures of neuronal progenitor cells lacking microglia have decreased proliferation as compared to cultures containing microglia, and notably, this phenotype was rescued when microglia were added to the culture medium (15). Additionally, it has been shown that inactivating microglia in the developing mouse cortex on postnatal day 5 resulted in increased neuronal apoptosis in layer 5 of the cerebral cortex (16). These studies imply that absence of trophic or other factors released by microglia have the potential to significantly alter the number of neurons within a specific brain region and therefore change the normal developmental trajectory of that region.

In addition to providing trophic support to developing neurons, it has also been shown that microglial cells play a central role in the normal postnatal apoptosis and phagocytosis of neurons and their connections, which are naturally over-produced and then “pruned” away based on experience-dependent usage (17). Specifically, numerous studies over the last decade have shown that microglia are essential in both initiating cell death and phagocytizing dying neurons in the developing retina, spinal cord, cerebellum, hippocampus, and cerebral cortex (12, 18). It is not completely understood how exactly microglia carry out this process of cell death; however, it is thought to involve the release of nerve growth factors, the production of superoxide ions, and/or the increased expression of microglial surface proteins CD11b and DAP12, which initiate signal transduction leading to apoptosis (19). Complement activation has also been implicated in the mechanism of synaptic pruning in microglia (20–22). This idea is supported by data showing that disruption of CR3/C3 signaling leads to increases in synapse numbers and connectivity (23). Interestingly, we previously found differential expression of *DAP12* in the prefrontal cortex (PFC) of postmortem brain tissue from children with ASD as compared to controls (24). Additionally, it is known that mutations in *DAP12* lead to Nasu-Hakola disease (OMIM# 221770), a rare autosomal recessive disorder characterized by bone abnormalities and adult-onset neuropsychiatric features, such as social disinhibition, distractibility, and lack of appropriate emotionality (25).

Apart from directly impacting the number of neurons in the developing brain, microglial cells have been shown to be involved in more nuanced aspects of synaptogenesis, such as in controlling the number of synapses, and in regulating synapse maturation and function (26–28). Synapse pruning is thought to be regulated by both spontaneous- and experience-driven processes, and recent studies have shown that microglia may be involved in both of these mechanisms. It has been shown that microglial cells contact dendritic spines, presynaptic terminals, and synaptic clefts during a critical period in the development of the mouse visual cortex, and additionally that the size of dendritic spines change after contact with microglia (29). Perhaps the most substantial mechanistic linkage of microglia to normal neurodevelopment and behavior was a study that assessed for both cellular and behavioral outcomes of mice lacking the Fractalkine receptor CX3CR1 that is expressed exclusively on microglia in the CNS (26). This study showed that *CX3CR1* knockout mice had delayed synaptic pruning, resulting in excessive and

electrophysiologically immature synapses. Moreover, related studies have even suggested that the behavioral phenotype of these mice is altered. For instance, it was demonstrated that mice lacking CX3CR1 had decreased functional brain connectivity, deficits in synaptic pruning, and behavioral changes associated with the autism phenotype, such as deficits in social interaction and increased repetitive behaviors (30).

Microglia in ASD

Although microglia's role in normal neurodevelopment is beginning to be well-recognized, parallel research into the role of microglia in autism and other neurodevelopmental disorders apart from their classic inflammatory function is only in its infancy. However, there is a large body of literature assessing aberrant immune function in peripheral tissue of children with ASD. For instance, numerous studies have suggested the innate immune response is globally abnormal in autistic patients (31–34). Specifically, peripheral blood monocytes have been shown to differ significantly in autistic patients. For instance, they secrete a cytokine pattern upon stimulation that is altered and more responsive to certain TLR ligands (35). Autistic patients also have higher plasma levels of factors involved with normal macrophage/monocyte activation, such as macrophage inhibitory factor and neopterin, and some studies have shown increased absolute monocytes on standard complete blood count in children with ASD as compared to controls (36, 37). Additionally, chemokines were shown to be increased in the plasma of children with ASD compared to age-matched typically developing controls and children with developmental disabilities other than ASD, and this increased chemokine production was associated with higher aberrant behavior scores and more impaired developmental and adaptive function (33). However, only more recently have rigorous assessment of microglia in postmortem brain tissue from patients with ASD been undertaken.

In 2005, Vargas and colleagues demonstrated that postmortem brain tissue from patients with autism exhibits an increased microglial density in gray matter and an activated microglial morphology; additionally, altered cytokine profiles were found in both ASD postmortem brain tissue and cerebrospinal fluid (7). Since this study, multiple other groups have reported increased microglial cell density in postmortem autism brains (8, 10), and we also reported increased numbers of microglial cell-specific surface markers in postmortem autism PFC as compared to control brains (24). Notably, the cellular volume of the activated microglia is two to four times the volume of quiescent microglial cells, and thus the increase in cell density may be partly related to increases in the number of activated microglia not overall microglial cell number alone (8). Additionally, it has been proposed that an increase in activated microglia may explain the increase in head circumference and brain volume observed in young children with autism (38), although this remains controversial as does the finding of increased head circumference in ASD in general (39).

In addition to cell-level studies of microglia in autistic postmortem brain tissue, a number of whole-genome expression studies have investigated RNA expression patterns in ASD postmortem brain tissue as compared to neurotypical controls (40–42). A recurrent finding in these studies is alterations in functional gene

ontology pathways related to the immune response. For instance, dysregulated levels of immune system-related genes have been demonstrated in multiple independent studies that have assessed many different brain regions (41–46). While these studies show that genes related to “immune” functions are aberrantly expressed in postmortem brain tissue from patients with ASD, they do not clarify whether underlying genetic variants cause this expression profile or if this pattern represents an epigenetic response to either endogenous or exogenous brain insults. Future work assessing for enrichment of genetic variants in genes exclusively expressed in microglia among patients with ASD would help answer this question and could be undertaken readily. Studies of epigenetic changes in individual cell types from postmortem autistic brain tissue may yield additional critical insight into the relative contribution of environmental factors on this repeatedly demonstrated gene expression profile.

While postmortem brain studies have begun identifying abnormal microglia in ASD, by their nature, they are inherently limited in attempting to ascertain if abnormal microglia are a reaction to, or the cause of, abnormal neurodevelopment. However, recent studies in rodent models of autism have begun to explore this question in more mechanistic detail. For instance, it was found that microglia from *MeCP2* null mice, a model of Rett syndrome, produced a conditioned media that damaged synaptic connectivity *via* a glutamate-excitotoxicity mechanism (9). Importantly, much of the phenotype associated with this disorder was reversed after transplanting wild-type microglia with a functional *MeCP2* gene, suggesting microglia play a key role in the pathogenesis of this disease. Moreover, it was recently demonstrated that the Rett phenotype may be partly related to *MeCP2* direct modulation of microglial inflammatory gene transcription (47). Similarly, a large body of work has demonstrated that maternal inflammation during gestation may result in phenotypes similar to ASD in both primates and rodents (48–50). This important work begins to bridge the gap in understanding between inherited risk for ASD and environmental exposures; yet, a mechanistic understanding of this work that relates to known postmortem findings in humans with ASD is not yet clear nor does it fully encompass the known genetic risk for ASD. Our hypothesis presented here would be further supported by these studies, in that the inherited risk for ASD could result in “primed” abnormal microglial cells that in individuals exposed to maternal inflammation or other factors results in an exaggerated/abnormal microglial response that perturbs normal neural network development (48).

Much work remains to definitively link abnormal microglia with the broad autistic phenotype; however, the evidence presented provides strong support to the notion that microglial cells may be able to reconcile the two most consistent gene expression and cellular findings in autism – changes in synaptogenesis and

immune pathways. This hypothesis is particularly exciting as inherent “hypomorphic” abnormalities in microglia at the genetic or epigenetic level and their resultant impaired functioning in neurodevelopment may help to explain the substantial contribution to autism risk that is thought to arise from interactions among intrinsic and environmental factors (51).

CONCLUSION

In summary, mounting evidence suggests that normal neurodevelopment represents a complex interplay between microglial cells and synaptic wiring. Furthermore, non-inflammatory roles for microglia in normal neurodevelopment are becoming increasingly identified. In parallel, abnormal immune signaling and microglial function are consistently demonstrated in postmortem tissue from autistic individuals as well as in mouse models of ASD. Previous research into altered immune function in autism has focused on the notion that microglia may cause destruction of already developed neural networks, perhaps in response to external insults, through their traditional inflammatory role. However, as their critical non-inflammatory role in normal neurodevelopmental becomes increasingly recognized, it is equally likely that this is what may be perturbed in ASD, yet was traditionally considered to be “immune dysregulation.” Future research should attempt to integrate the genetic and epigenetic etiology, neuronal synaptic disconnectivity, and abnormal microglia/immune findings in autism in an attempt to determine the precise role non-immune functions of microglia may play in the pathogenesis of ASD and other neurodevelopmental disorders.

AUTHOR CONTRIBUTIONS

CAE and MNZ contributed equally to the conception, writing, and editing of the manuscript. OMR was involved in the conception and editing of the manuscript.

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Neuron–Microglia Interactions in Mental Health Disorders: “For Better, and For Worse”

Eric S. Wohleb^{1,2*}

¹ Department of Psychiatry, Yale University School of Medicine, New Haven, CT, USA, ² Department of Psychiatry and Behavioral Neuroscience, University of Cincinnati College of Medicine, Cincinnati, OH, USA

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Edited by:

Sanae Hasegawa-Ishii,
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National Institute for Basic Biology,
Japan
Kazuhiko Watabe,
Kyorin University, Japan

*Correspondence:

Eric S. Wohleb
eric.wohleb@uc.edu

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Persistent cognitive and behavioral symptoms that characterize many mental health disorders arise from impaired neuroplasticity in several key corticolimbic brain regions. Recent evidence suggests that reciprocal neuron–microglia interactions shape neuroplasticity during physiological conditions, implicating microglia in the neurobiology of mental health disorders. Neuron–microglia interactions are modulated by several molecular and cellular pathways, and dysregulation of these pathways often have neurobiological consequences, including aberrant neuronal responses and microglia activation. Impaired neuron–microglia interactions are implicated in mental health disorders because rodent stress models lead to concomitant neuronal dystrophy and alterations in microglia morphology and function. In this context, functional changes in microglia may be indicative of an immune state termed parainflammation in which tissue-resident macrophages (i.e., microglia) respond to malfunctioning cells by initiating modest inflammation in an attempt to restore homeostasis. Thus, aberrant neuronal activity and release of damage-associated signals during repeated stress exposure may contribute to functional changes in microglia and resultant parainflammation. Furthermore, accumulating evidence shows that uncoupling neuron–microglia interactions may contribute to altered neuroplasticity and associated anxiety- or depressive-like behaviors. Additional work shows that microglia have varied phenotypes in specific brain regions, which may underlie divergent neuroplasticity observed in corticolimbic structures following stress exposure. These findings indicate that neuron–microglia interactions are critical mediators of the interface between adaptive, homeostatic neuronal function and the neurobiology of mental health disorders.

Keywords: neuroplasticity, microglia, anxiety, depression, post-traumatic stress disorder, neuroinflammation, parainflammation, neuroimmune

INTRODUCTION

Mental health disorders, such as anxiety and depression, are a major source of disability leading to significant social and economic burden throughout the world (1–3). While progress has been made in understanding the neurobiology of mental health disorders, it is clear that multiple subtypes exist with varied pathophysiological mechanisms (4). Clinical and preclinical studies show that anxiety- and depressive-like symptoms are linked to altered neuroplasticity in corticolimbic

brain regions. In particular, divergent responses are reported; neuronal atrophy and synapse loss in the prefrontal cortex and hippocampus, and neuronal hypertrophy and increased synaptic density in the amygdala and nucleus accumbens (5). Moreover, clinical studies show that many anxiety and depressive symptoms develop or worsen following exposure to psychosocial and environmental stress (6–8). This is pertinent as rodent models of stress can provide insight into the mechanisms that contribute to the neurobiology of anxiety- and depressive-like behaviors. Indeed several stress models, including repeated social defeat and chronic unpredictable stress, recapitulate key neuroplasticity changes that contribute to susceptibility and development of anxiety- and depressive-like behaviors (9–11).

Coinciding with altered neuroplasticity, repeated stress exposure leads to dysregulation of neuroimmune systems that are implicated in mental health disorders (12–15). Microglia are tissue-resident macrophages in the brain that integrate stress-induced neuroimmune signals leading to behavioral consequences (16). Following stress exposure, microglia undergo dynamic alterations in morphology and function within corticolimbic brain regions implicated in anxiety- and depressive-like symptoms (17). In line with these findings, recent studies demonstrate that microglia have an integral role in shaping neuronal responses (i.e., activity) and synaptic elements (i.e., dendrites and dendritic spines) (18), which support adaptive behavior and cognition (19). Thus, stress-associated changes in neuron–microglia interactions may play an integral role in the pathophysiology of mental health disorders. Clinical studies using histological analyses suggest that microglia have altered morphology and function in depressed individuals (20, 21). Moreover, a clinical neuroimaging study showed that individuals experiencing a major depressive episode have enhanced positron emission topography (PET) labeling of the translocator protein (TSPO), a putative marker of neuroinflammation and microglia activation (22). Further studies using microarray gene expression analyses demonstrate increased cytokine and complement pathways in the PFC and hippocampus of postmortem samples obtained from depressed individuals (23, 24). In contrast, clinical reports examining cerebrospinal fluid showed no changes in immune-related markers in depressed patients (25, 26), suggesting that neuroimmune dysregulation may represent a pathophysiological mechanism in a subset of depressed patients. Notably, clinical data showed that individuals with atypical depression had higher levels of inflammatory markers in circulation compared to controls and those with melancholic depression (27). Altogether, clinical studies and preclinical models provide evidence that neuroimmune alterations provoked by stressors may contribute to the neurobiology of mental health disorders in a subset of individuals.

Concomitant neuronal dystrophy and microglia activation implicate reciprocal neuron–microglia interactions in behavioral deficits, and these responses may not directly lead to neuroinflammation *per se*. As researchers demonstrate the ever-changing form and functions of microglia, it is clear that morphological characteristics may reflect several phenotypes. Thus, microglia appear to display specialized responses that are brain region-dependent and dictated by the stress model and duration. These brain region-specific microglia phenotypes may play a role in

divergent neuroplasticity observed in corticolimbic brain regions following stress exposure (28). It is also important to note that stress-induced neuroimmune alterations are modest compared to other pathological situations (29). These neuroimmune processes mediated by microglia do not generally lead to neurotoxicity but may contribute to neuronal dystrophy following stress. In this context, stress-associated functional changes in microglia may be indicative of an immune response similar to parainflammation (30), rather than “neuroinflammation.” This notion of stress-induced parainflammation in the brain will be discussed further in this review. Here, literature are presented showing that neuron–microglia interactions have an integral role in promoting homeostasis in the brain as well as how perturbations in neuron–microglia interactions lead to impaired neuroplasticity. In particular, this review will focus on evidence suggesting that repeated stress exposure leads to dysregulated neuron–microglia interactions and neuroplasticity deficits with implications for mental health and neurological disorders.

NEURONS REGULATE MICROGLIA FUNCTION

The mammalian central nervous system develops circuits of interconnected neurons that underlie complex functions, including cognition and behavior (31–33). Several cell types residing in the brain contribute to the development and maintenance of this neurocircuitry. Important cellular counterpart of neurons are microglia, which are brain-resident macrophages that direct homeostatic functions and mediate immune responses to pathological conditions (13, 34). Microglia are distributed throughout the brain but appear to have varied roles in specific regions and develop unique features based on tissue-specific molecular signals (28, 35, 36). Recent studies reveal that microglia are maintained in the brain through self-renewal (37, 38), suggesting that these long-lived brain-resident macrophages maintain long-term interactions with proximal neurons. Neuron–microglia interactions are mediated by soluble factors as well as contact-dependent mechanisms (39, 40), and reciprocal communication is necessary for adaptive neuroplasticity and behavior. These features place microglia as a critical mediator of neuronal function for better, and for worse (**Figure 1**).

Neurons regulate microglia function through soluble factors, including chemokines, cytokines, and neurotransmitters (**Figure 1**). Neuron-derived fractalkine (CX₃CL1) regulates microglia activation through binding to CX₃CR1, which is enriched on microglia (41). CX₃CL1 is expressed by neurons in two forms (membrane-bound or cleaved soluble proteins) that may transduce different molecular signals (42). The CX₃CL1–CX₃CR1 pathway is shown to be critical for proper neurodevelopment, and the absence of this pathway significantly impaired brain connectivity leading to social interaction deficits (43–45). In addition, recent work shows that microglia develop a unique phenotype based on soluble molecular cues likely derived from proximal neurons. For instance, transforming growth factor (TGF)- β promotes a unique transcriptional profile that organizes transcriptional pathways within microglia (35, 36). Other studies

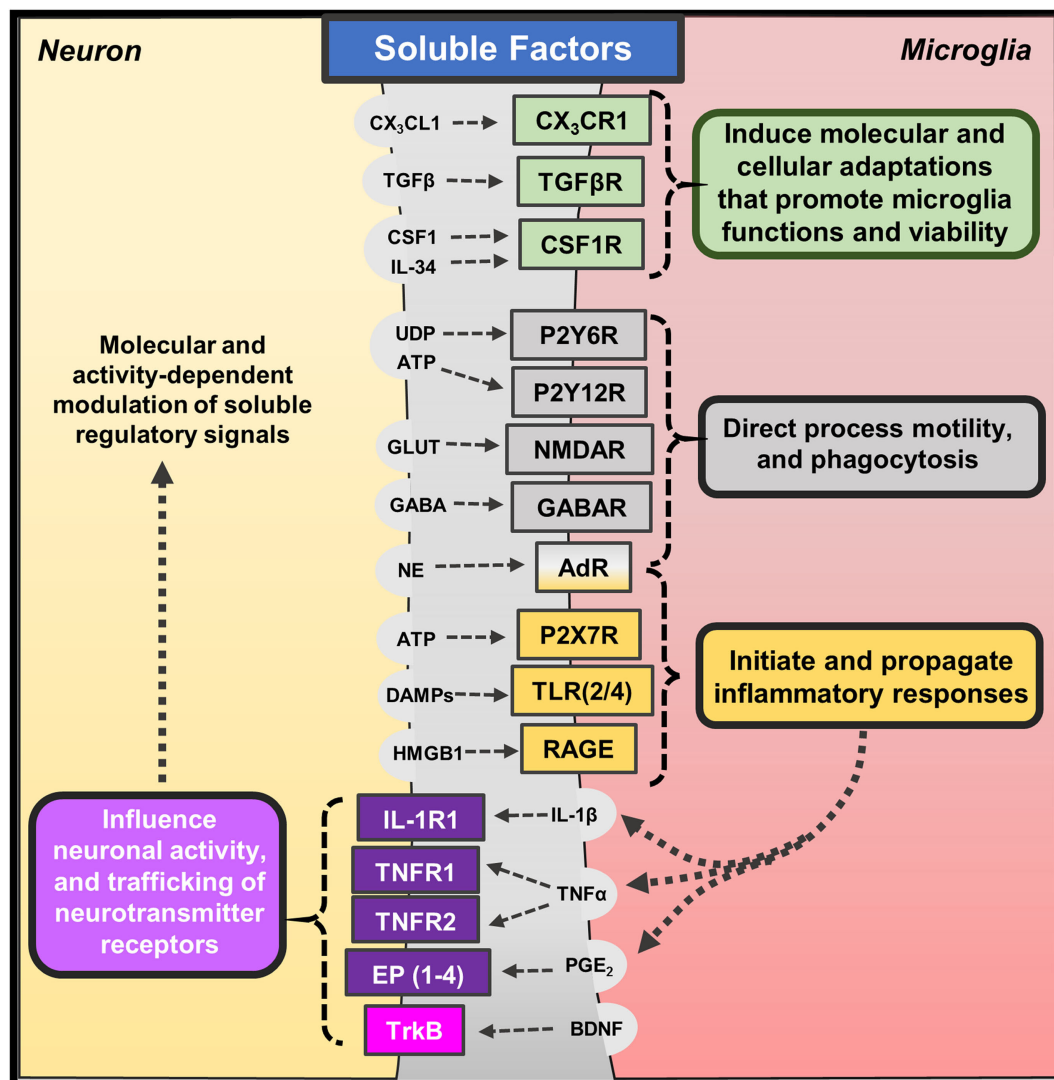


FIGURE 1 | Soluble factors regulating neuron–microglia interactions. Several molecular and cellular pathways mediate neuron–microglia interactions. Neurons release soluble immune-related factors (fractalkine – CX₃CL1; transforming growth factor-β – TGFβ; colony-stimulating factor-1 – CSF1; interleukin-34 – IL-34), nucleotides (uridine diphosphate – UDP; adenosine triphosphate – ATP), neurotransmitters (glutamate – GLUT; γ-aminobutyric acid – GABA; norepinephrine – NE), and danger- or damage-associated molecules (DAMPs; high mobility group box 1 – HMGB1) that bind to cognate receptors on microglia. These soluble neuron-derived signals promote many homeostatic functions but can also initiate or propagate neuroinflammation depending on the context. Microglia also release soluble factors, such as cytokines (interleukin-1β – IL-1β; tumor necrosis factor-α – TNFα), prostaglandins (prostaglandin E₂ – PGE₂), and neurotrophins (brain-derived neurotrophic factor – BDNF), which bind to neuronal receptors. Microglia often release these factors in response to fluctuations in neuron-derived signals; yet, low levels of microglia-derived cytokines are shown to promote homeostatic neuroplasticity through trafficking of neurotransmitter receptors.

show that colony-stimulating factor (CSF)-1 and interleukin (IL)-34 signal microglia through CSF1 receptor to regulate their development and viability (46). Indeed, microglia do not develop in mice lacking CSF1 receptor (47), and recent work shows pharmacological blockade of CSF1 receptor caused depletion of microglia in the brain (37). These studies highlight some of the soluble neuron-derived signals that shape microglia morphology and function.

The distinct, surveying phenotype of microglia allows them to rapidly respond to perturbations in their microenvironment

along with deviations in neuronal homeostasis and activity (48). These responses are mediated, in part, by neuronal release of nucleotides (i.e., UDP, ATP) (49) and neurotransmitters [i.e., glutamate, GABA, and norepinephrine (NE)] (19). Indeed, acute glutamate uncaging induced microglia chemotaxis and convergence of microglia processes toward sites of increased neuronal activity (50). Other studies show that microglia morphology and process motility is influenced by extracellular glutamate, GABA, and NE (51). The chemotactic properties of ATP appear to be predominantly mediated by microglia expression of P2Y12 receptor.

For instance, microglia lacking P2Y₁₂ receptors display similar baseline surveillance of regional microenvironments; however, their motility is impaired during injury responses when nucleotides are released (52). Similar reports suggest that fluctuations in calcium signaling along with ATP signaling through P2Y₁₂ receptors lead to microglia process convergence as well (53). Interestingly, a recent study indicated that microglia expression of P2Y₁₂ receptors is required for proper development of visual cortex and ocular dominance (54). Other purinergic receptors, such as P2Y₆, may play a functional role in these responses as UDP signaling through P2Y₆ receptors increased microglia-mediated phagocytosis following hippocampal excitotoxicity (55). These findings indicate that nucleotides released following neuronal activity act as attractants for microglia processes, and dysregulated neuroplasticity is observed in the absence of these interactions.

MICROGLIA DIRECT AND SHAPE NEURONAL FUNCTION

Microglia release soluble factors, including cytokines and prostaglandins, which reciprocally influence and modulate neuronal function (Figure 1). There are significant primary research reports and reviews that describe how cytokines and other immune mediators released by microglia influence neuronal function and neuroplasticity. Indeed, low levels of IL-1 β are required for long-term potentiation, while basal levels of TNF α are necessary for proper homeostatic trafficking of AMPA and GABA_A receptors, termed synaptic scaling (56, 57) [see also reviews, Ref. (58, 59)]. Based on these studies, it

is evident that microglia-mediated cytokine and prostaglandin synthesis can modulate neuronal responses during physiological and pathological conditions. Further studies indicate that microglia-derived cytokines can indirectly affect neurons through gliotransmission mediated by astrocytes (60). For instance, the release of TNF α by activated microglia potentiated astrocyte glutamate release, which can modulate synaptic plasticity and even lead to neurotoxicity (61). In addition, ATP released by microglia is shown to induce glutamate release by astrocytes thereby acutely exciting proximal neurons (62). These indirect signaling pathways may be further augmented during inflammatory conditions. For example, microglia activation and TNF α release increased neuronal hyperactivity and susceptibility to develop seizures (63). Other data show that microglia can support adaptive synaptic plasticity through the release of neurotrophic factors, such as brain-derived neurotrophic factors (BDNF) (64). These findings are not entirely surprising because microglia as the tissue-resident macrophages of the brain mediate pathological processes along with reparative or growth responses (65).

Recent studies also reveal that microglia direct neuronal function through contact-dependent mechanisms, including engulfment of synaptic and dendritic elements (18, 19) (Figure 2). Seminal studies showed that microglia actively phagocytose synapses during neurodevelopment (66). Synapse elimination during neurodevelopment is mediated by complement factor 3 and 1b, which bind synapses with diminished activity, initiating microglia-mediated phagocytosis *via* complement receptor 3 (CR3; CD11b) (67). Other formative studies showed that microglia perform regular, but brief “synapse sampling” by contacting synapses in the adult brain. Of note, *in vivo* imaging showed

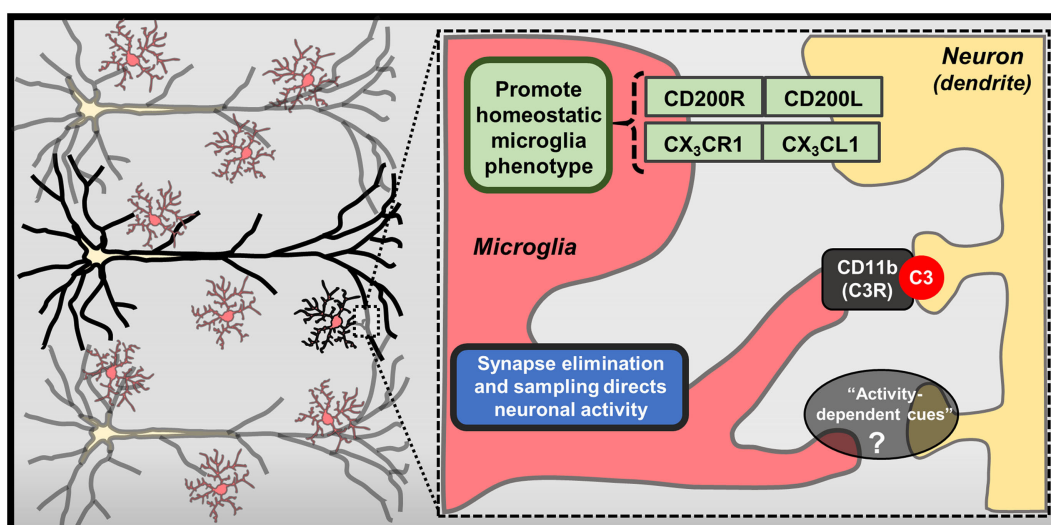


FIGURE 2 | Contact-dependent mechanisms mediating neuron–microglia interactions. Physical interactions between neurons and microglia exist, including membrane-bound CX₃CL1 and CD200L, which bind CX₃CR1 and CD200R on microglia, respectively. Further recent studies have highlighted specialized mechanisms that contribute to contact-dependent synaptic modulation, such as binding of complement component C3 to synapses and eventual removal of these “tagged” synapses by microglia through CD11b/CR3-mediated phagocytosis. Microglia can also envelop hyperactive neurons and regularly perform “synapse sampling” *via* activity-dependent mechanisms that are not entirely defined (?).

that microglia sampled proximal synapses once per hour and were drawn more frequently to active synapses. Moreover, these studies show that prolonged microglia interactions resulted in synapse loss (68). Other studies show similar activity-dependent microglia-mediated synapse elimination occurs in cortical brain regions (69). In these studies, soluble factors that modulate neuron–microglia interactions may play a prominent role as purinergic signaling along with release of neurotransmitters that rapidly draw microglia processes toward elevated neuronal activity. For instance, glutamate uncaging caused attraction of microglia processes, which subsequently surrounded hyperactive neurons, leading to contact-dependent reductions in neuronal activity (50). While it is clear that microglia can physically remove synapses, it remains to be determined what mechanisms contribute to microglia-mediated reductions in neuronal activity (**Figure 2**). These findings demonstrate that microglia are directed by soluble neuron-derived cues to initiate contact-dependent regulation of neuronal activity.

To further examine the functional role of microglia in various physiological and pathological conditions, several groups have developed methods to deplete microglia (70). Initial studies provide compelling evidence that microglia are necessary for adaptive neuroplasticity and behavior. For instance, mice treated with clodronate liposomes showed robust microglia depletion in the hippocampus, which led to spatial memory decrements as well as reduced sociability. These cognitive and social deficits were recapitulated with widespread microglia depletion using the CSF1 receptor antagonist PLX3397. Of note, cognitive and behavioral consequences of microglia depletion were attenuated following repopulation (71). Other studies revealed that clodronate depletion of microglia resulted in enhanced synapses and excitatory input on hippocampal neurons (72). These neurobiological effects have functional implications as pharmacogenetic microglia depletion caused impairments in the rotarod motor learning task (64). These deficits in motor learning were recapitulated when BDNF expression was selectively deleted from microglia. Despite these findings, other studies indicate that widespread microglia depletion with PLX3397 caused no significant alterations in cognition or behavior (37). The dynamic function of microglia in these depletion studies likely reflects their compartmentalized brain region-specific functions (28, 73, 74). These unique neuron–microglia interactions highlight the complexity of molecular and cellular pathways that regulate neurobiology and behavior.

In the end, reciprocal neuron–microglia interactions are regulated by soluble and contact-dependent pathways. These pathways enable microglia to obtain feedback on neuronal functions and rapidly enact interventions to maintain tissue homeostasis. These neuron–microglia interactions appear to support neuronal homeostasis because perturbations in these pathways often result in neuroplasticity impairments and influence performance in memory-based tasks (**Figures 1 and 2**). In this context, neuron–microglia interactions may be disrupted during pathological conditions, such as mental health and neurological diseases. Further studies will be reviewed to provide evidence that neuron–microglia interactions may play a critical role in the neurobiology of mental health disorders.

PSYCHOSOCIAL AND ENVIRONMENTAL STRESSORS CAUSE CONCOMITANT NEURONAL DYSTROPHY AND MICROGLIA ACTIVATION

Exposure to psychosocial and environmental stress is shown to cause robust neuronal activation (i.e., cFos, FosB) through the release of glutamate and NE in corticolimbic brain regions, such as the prefrontal cortex, amygdala, and hippocampus (75–78). Further perceived perturbations of homeostasis caused by stress lead to neuroendocrine activation and release of glucocorticoids (GC) into circulation (79). Converging lines of evidence indicate that aberrant neuronal activation coupled with elevated GC levels lead to neuronal dystrophy in corticolimbic brain regions following stress (**Figure 3**). For instance, repeated stress caused dendritic atrophy and synapse loss on pyramidal neurons in the rat prefrontal cortex (80, 81), and these effects were recapitulated with chronic GC administration (82). Stress-induced atrophy of pyramidal neurons may be related to impaired glutamate receptor (i.e., NMDA, AMPA) expression in the prefrontal cortex, which is dependent on GC receptor activation (83). Recent work also suggests that dysregulation of interneurons may contribute to disrupted microcircuitry in the prefrontal cortex and depressive-like behavior (84, 85). Together, these neurobiological alterations contribute to shifted excitatory–inhibitory tone in the prefrontal cortex, which is proposed to underlie anxiety and depressive symptoms (9, 86, 87) (**Figure 3**). Further, brain regions that receive PFC projections, including amygdala, hypothalamus, hippocampus, and nucleus accumbens, exhibit disrupted function as well (88, 89).

Corresponding with altered neuroplasticity in these corticolimbic regions, several reports demonstrate changes in microglia morphology in overlapping brain regions following stress (**Figure 3**). Indeed, morphological changes in microglia are observed in the prefrontal cortex, amygdala, hypothalamus, hippocampus, and nucleus accumbens following stress exposure (16, 17, 90). Several neuronal and endocrine pathways are shown to cause microglia activation in rodent models of stress. For instance, early work showed that noradrenergic pathways in the brain contribute to elevations in stress-induced pro-inflammatory cytokine expression (91, 92). Further work showed that blockade of β -adrenergic receptors with propranolol prevented microglia activation and pro-inflammatory cytokine gene expression in enriched microglia following repeated social defeat (93). Using inescapable footshock stress, Frank et al. showed that RU486 can prevent stress-induced microglia priming in the hippocampus, suggesting that GCs contribute to these neuroimmune responses as well (94–96). Similar studies using repeated restraint stress showed that pharmacological blockade of NMDA receptors or GC receptors can attenuate stress-associated microglia proliferation, providing evidence that microglia responses are caused by aberrant neuronal activation and neuroendocrine responses (97). In contrast to these findings, recent work by Yirmiya and colleagues showed that chronic stress exposure caused microglia to undergo atrophy in the dentate gyrus of the hippocampus, and these deficits were associated with depressive-like behaviors

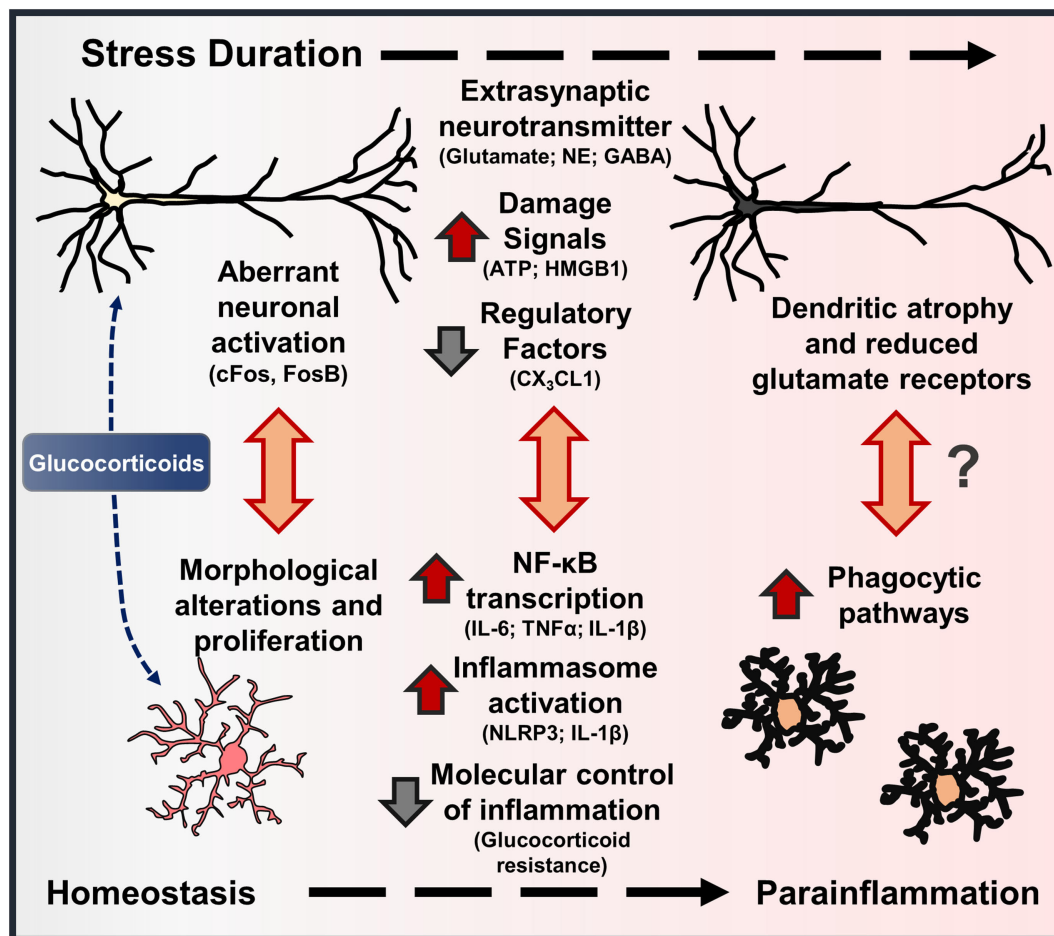


FIGURE 3 | Stress-induced neuronal dystrophy contributes to alterations in microglia function and parainflammation. Models of environmental and psychosocial stress activate characteristic neuroendocrine (i.e., glucocorticoids – GC) and neuroimmune pathways that contribute to neuroplasticity alterations underlying anxiety- and depressive-like behaviors. In this cascade, there are features that show stress-induced “neuroinflammation” resembles parainflammation. As brain-resident macrophages, microglia interact with proximal neurons, and disruptions in neuron–microglia interactions initiate morphological and functional changes in microglia. Repeated stress (i.e., prolonged elevations in glucocorticoids) leads to aberrant neuronal activation, extrasyaptic neurotransmitter levels, and release of neuron-derived damage signals. These signals further promote mild to moderate levels of neuroinflammatory transcription and inflammasome activation in microglia. With persistent or chronic stress, pyramidal neurons undergo dendritic atrophy and reduce glutamate receptors, which may be precipitated by cytokines released by proximal microglia or microglia-mediated synapse elimination. Collectively, these stress-induced neuronal responses and resultant molecular and cellular cascades resemble a state of parainflammation.

(98). Further peripheral administration of agents that stimulate microglia function (i.e., LPS, CSF1) reversed microglia atrophy and provided antidepressant effects (98). These compelling findings suggest that brain region-specific signals lead to microglia dysfunction, which can have deleterious effects on neurobiology and behavior as well. Collectively, these studies indicate that dynamic, brain region-specific functional changes in microglia are driven by stress-induced neuroendocrine and neurotransmitter pathways.

Stress-induced microglia activation may also stem from increased release of danger-associated molecular patterns (DAMPs) that bind pattern recognition receptors (PRR) to promote neuroinflammatory signaling through activation of the NLRP3 inflammasome (99). For instance, stress-induced

release of the DAMP, high mobility box group (HMGB)-1, caused microglia priming through inflammasome activation and increased pro-inflammatory gene expression (100). In other studies, repeated stress is shown to increase tissue IL-1β levels and depressive-like behaviors; these neuroimmune and behavioral responses are prevented in mice lacking the NLRP3 inflammasome (101, 102). This is consistent with recent work that showed during immobilization stress there is influx of extracellular glutamate and ATP in the hippocampus, followed by elevations in IL-1β and TNFα. Of note, increased release of IL-1β and TNFα is blocked by administration of P2X7 receptor antagonist. In addition, follow-up studies showed that stress-induced depressive-like behaviors were blocked by the P2X7 receptor antagonist and in mice lacking the NLRP3 inflammasome (103).

Other reports suggest that increased NLRP3 activation and IL-1 β levels in the prefrontal cortex are mediated by microglia following chronic stress, and these responses are reversed with chronic fluoxetine treatment (104). These studies support previous work showing that chronic stress promoted elevations in IL-1 β signaling, which contributed to reduced hippocampal neurogenesis and depressive-like behavior (105, 106). Another recent study showed that restraint stress increased TNF α levels in the hippocampus with delayed elevations of several inflammatory mediators in the amygdala, but not in the prefrontal cortex (107). As noted, neuron-derived CX₃CL1 acts as integral regulator of microglia function, and deficient signaling can result in neuroplasticity deficits (45). Thus, stress-induced reductions in CX₃CL1 may contribute further to microglia activation and anxiety- or depressive-like behaviors (108). These studies provide evidence that fluctuations in neuron-derived signals (i.e., ATP, HMGB1, and CX₃CL1) act to regulate microglia activation and subsequent production of pro-inflammatory cytokines with repeated stress exposure, which will influence neuronal function and behavior.

In line with these findings, recent studies show that anxiolytic and antidepressant treatments can block or reverse stress-induced microglia activation. For instance, administration of imipramine following repeated social defeat reduced IL-6 expression in enriched microglia and attenuated depressive-like behaviors following (109). This is consistent with other studies showing selective serotonin reuptake inhibitors produce anti-inflammatory responses in microglia (110). Further work using repeated social defeat demonstrated that pretreatment with benzodiazepines reduced markers of neuroinflammation and diminished anxiety-like behavior (109). Similarly, pretreatment with the non-selective β -adrenergic receptor antagonist propranolol prevented microglia activation and blocked the development of anxiety-like behavior following repeated social defeat (93). These findings raise the question whether these interventions that targeted neurotransmitter systems subsequently attenuated neuroimmune activation or if microglia were the primary effector. In the end, these results suggest that pharmacological treatments may produce anxiolytic or antidepressant effects by simultaneously normalizing neurotransmission and modulating microglia functions. Furthermore, these findings highlight the need to determine cell type-specific pathways that lead to neuronal dystrophy or microglia activation, respectively. As an example, stress-induced microglia activation can be prevented by GC blockade; however, this may be an effect of limiting neuronal dystrophy and diminished release of damage-associated factors or direct effects on microglia. Dissociating the molecular and cellular pathways that contribute to neuronal and microglia adaptations following repeated stress exposure may show novel pharmacological or molecular therapeutic targets.

STRESS-INDUCED “NEUROINFLAMMATION” RESEMBLES PARAINFLAMMATION

Based on the overlap of stress-induced neuronal dystrophy and functional changes in microglia, it is evident that stress-induced

“neuroinflammation” may reflect an inflammatory state termed parainflammation (30). The notion of parainflammation is related to the condition originally described as “physiological inflammation” by Élie Metchnikoff (111). Indeed, parainflammation is an immune state induced by “stressed or malfunctioning” tissues and is considered to be an intermediate phase between homeostasis and classical inflammation. Moreover, parainflammation appears to be mediated predominantly by tissue-resident macrophages, such as microglia. Thus, microglia activation following stress exposure and reported in depression may represent a state of parainflammation, in part, as a response to neuronal dystrophy. In support of this idea, it is evident that stress-induced microglia production of pro-inflammatory cytokines is modest compared to other pathological conditions (29). Moreover, aberrant neuronal activation and damage signals released during stress exposure appear to provoke microglia activation (99), which contributes to altered neuroplasticity and anxiety- or depressive-like behaviors. Stress duration and intensity likely influence the scale of parainflammation, and these prolonged impairments in neuron–microglia interactions may lead to irreversible neurobiological consequences and mental health disorders (Figure 2).

It is worthwhile to note that parainflammation is not considered an entirely detrimental state because it is provoked by malfunctioning cells, and the objective is to restore tissue homeostasis (112). There is evidence that microglia-mediated activation or “neuroinflammation” in non-pathological conditions may initiate adaptive functions to restore neuronal activity to basal, physiological levels. For instance, repeated peripheral endotoxin challenge caused microglia processes to interfere with inhibitory interneuron synaptic connections on pyramidal neurons in the cortex, which provided neuroprotection through increased neuronal activity (113). In separate studies microglia were shown to play a neuroprotective role during excitotoxic injury, with microglia depletion leading to enhanced susceptibility to neuronal death (114). Consistent with these studies, recent work showed that microglia processes contacted swollen axonal segments during excitotoxic conditions, which normalized the excitability of affected neurons and preserved their viability (115). In these instances, neuron–microglia interactions are critical to maintain homeostasis in the brain but likely lead to undesirable inflammatory consequences. It is critical to point out that in many pathological conditions inflammatory responses are required to limit neuronal death and promote tissue repair processes (65, 116). In this context, stress-induced parainflammation and alterations in microglia function may be aimed to restore neuronal homeostasis. It is possible that these processes are initially protective but may generate pathological consequences with chronic stress exposure. Further studies will need to be performed to establish the dynamic role of microglia in these neurobiological responses.

This is not a novel proposal as recent reviews have proposed that a subset of depressed individuals develop a chronic state of parainflammation that contributes to the pathophysiology underlying their symptoms (4, 117). Others have suggested similar models in which stress exposure causes neuronal microdamage, and neuroinflammatory responses are initiated by microglia

to promote repair (118). Depending on the neurocircuitry affected, it is argued that anxiety- or depressive-like symptoms may develop, suggesting that neuroinflammatory pathways may lead to divergent neurobiological changes (118). The neuronal microdamage model is compelling; however, further studies will need to be performed to determine molecular and cellular mediators of these effects. The specific molecular and cellular mediators of stress-induced parainflammation are particularly relevant in the context of potential therapies for mental health disorders. For instance, some common antidepressant drugs, such as selective serotonin reuptake inhibitors, have reported anti-inflammatory effects (119). In other cases, the antidepressant behavioral effects of serotonin reuptake inhibitors were blocked by anti-inflammatory drugs (120). These data demonstrate that dynamic neuron–microglia interactions modulate behavior and suggest that parainflammation may enact microglia-mediated mechanisms that normalize neuronal function. In either case, further studies will need to examine the neuron–microglia interactions that contribute to stress-induced parainflammation, and how interventions can engage these mechanisms to provide therapeutic benefits.

ROLE OF PERIPHERAL BONE MARROW-DERIVED MYELOID CELLS IN STRESS-INDUCED PARAINFLAMMATION

Another important mediator of neuroimmune functions are peripheral immune cells, which propagate immune signals in the brain and influence behavior. Indeed, studies indicate that specific behavioral issues, such as pathological grooming, can be attributed to peripheral hematopoietic immune cells (121). In the context of stress, recent evidence demonstrates a role of peripheral myeloid-derived cells (monocytes and granulocytes) in “neuroinflammation” and anxiety- or depressive-like behavior (14, 90). In these studies, social defeat increased monocyte recruitment to the brain through canonical chemokine pathways (CCL2–CCR2 and CX₃CL1–CX₃CR1) (108). Moreover, peripheral monocytes may be preferentially recruited to specific brain regions through adhesion molecule expression (122). In the context of neuroinflammation, it is plausible that this is an attempt to restore homeostasis as neuronal stress-associated signals reach upper limits of parainflammation (112). Initial studies show that stress caused peripheral monocytes to infiltrate the brain under defined conditions; however, further work revealed that peripheral monocytes do not significantly contribute to brain-resident microglia populations (123, 124). Further, pharmacological or genetic techniques to limit monocyte trafficking in the brain and neuroinflammation prevent social defeat-induced anxiety-like behaviors (14, 90). Together, these studies revealed that peripheral monocytes reinforce neuroinflammatory processes and highlight a novel neuroimmune axis that promotes mood disturbances (90, 125). Further studies will need to be performed using microglia- or monocyte-specific genetic techniques or depletion to distinguish cell type-specific contributions to reported neuroimmune mechanisms and their influence on neuronal responses.

BRAIN REGION-SPECIFIC MICROGLIA RESPONSES MAY UNDERLIE DIVERGENT NEUROPLASTICITY OBSERVED IN MODELS OF STRESS-INDUCED MENTAL HEALTH DISORDERS

Microglia are implicated in the neurobiology of several mental health disorders; however, it is unclear how they contribute to divergent neuroplasticity alterations in corticolimbic brain regions. It is plausible that brain region-specific neuron–microglia interactions contribute to divergent neuroplasticity reported. For instance, work by Hinwood and colleagues showed that the putative microglia inhibitor minocycline attenuated alterations in microglia morphology and reduced FosB activation in the medial prefrontal cortex, and prevented working memory deficits following repeated stress exposure (126). These results suggest that microglia activation may modulate neuronal responses and cognitive deficits in a rodent stress model. While there is limited clinical evidence showing microglia modulation of neurons, a recent study showed that elevated C-reactive protein levels in circulation, indicative of low grade peripheral inflammation, is associated with increased glutamate levels in the basal ganglia of depressed patients (127). As noted, microglia may modulate neurobiological and behavioral consequences of psychological stress through the release of pro-inflammatory cytokines (i.e., IL-1 β , TNF α , and IL-6) (13, 14, 16). Indeed pro-inflammatory cytokines released by microglia following repeated stress exposure may produce brain region-specific alterations in synaptic plasticity. For instance, TNF α administration on pyramidal neurons derived from the hippocampus increased AMPA receptor trafficking to postsynaptic sites and concomitant reductions in GABA_A receptors that caused increased excitability (57, 128). In contrast, TNF α administration on striatal brain slices caused reduced AMPA receptor levels on medium spiny neurons (129). Further TNF α released from microglia led to reduced excitability of medium spiny neurons in the striatum after cocaine administration (130). In these studies, the actions of TNF α are mediated by TNF receptor 1 expressed on neurons, thus varied neurophysiological effects may be dependent on neuron subtype-specific molecular signaling (128). Separate studies showed repeated social defeat-induced microglia-mediated prostaglandin release that attenuated neuronal responses in the ventral tegmental area (VTA). The reduced firing of VTA neurons following repeated social defeat increased social avoidance, indicating that microglia-mediated modulation of this mesolimbic neuronal pathway contributed to the development of depressive-like behavior (131). These findings provide compelling evidence that microglia produce soluble factors eliciting brain region-specific neuronal responses that elicit cognitive and behavioral consequences.

Other studies show that contact-dependent neuron–microglia interactions are critical modulators of neuronal and behavioral responses to stress. In particular, recent work showed that microglia-mediated elimination of synaptic elements may contribute to stress-induced synaptic plasticity deficits in the hippocampus. For instance, 14 days of chronic unpredictable stress increased

the presence of dendritic and synaptic elements in the processes of microglia in the CA1 of the hippocampus. The increased presence of neuronal elements in microglia was associated with reduced sucrose preference and impaired long-term potentiation. Moreover, mice lacking CX₃CR1 were resilient to CUS, which corresponded with reduced microglia phagocytosis of neuronal elements, attenuated LTP deficits, and normalized sucrose preference (132). It is important to reiterate that mice lacking CX₃CR1 have delayed neurodevelopment and display baseline social interaction deficits (43, 44), which may contribute to observed stress resilience. It is unclear if microglia expression of CX₃CR1 is necessary for stress-induced activation, but other studies provide evidence that neuronal CX₃CL1 activated microglia, which led to the modulation of synaptic strength. For instance, CX₃CL1 binding to CX₃CR1 on microglia caused increased IL-1 β release and downstream molecular mechanisms that altered synaptic plasticity (133). Further it is important to consider that microglia lacking CX₃CR1 may be diverted away from homeostatic functions, such as clearance of neural progenitors in the hippocampus, which may exacerbate stress-induced neuropathology (134, 135). These studies demonstrate that microglia can shape neuronal responses to stress through contact-dependent mechanisms, contributing directly to the development of depressive-like behaviors.

The mechanisms that govern microglia-mediated synapse interactions during stress exposure have not been extensively studied; however, as noted microglia are drawn to synapses in an activity-dependent manner. This is pertinent as repeated stress exposure is known to increase neuronal activity in several corticolimbic brain regions, and these neuronal networks are dysregulated in mental health disorders (87, 89). In this context, stress-associated neuronal hyperactivity in specific brain regions may elicit microglia-mediated disruption of synaptic connections with unintended consequences. For instance, microglia disruption of inhibitory synapses on pyramidal neurons may lead to aberrant neuronal activity and extrasynaptic glutamate neurotransmission (136). The potential microglia-mediated exclusion of inhibitory synapses may have consequences on the integrity of interneurons as well. Indeed, recent work indicates that cortical interneurons may be more susceptible to stress-induced dystrophy (85). It is unclear if interneurons are susceptible to stress-induced parainflammation, but deep sequencing techniques in neuron subtype-specific populations may lend insight (137). Further studies will need to expound on these findings to determine how microglia facilitate or disrupt neuroplasticity in stress-responsive brain regions.

In all, microglia have dynamic brain region-specific functions that may contribute to divergent neuroplasticity effects underlying stress-induced anxiety- and depressive-like behavior (5, 13). As the roles of microglia expand, it will be important to characterize brain region-specific phenotypes (28). These studies will undoubtedly reveal unique microglia properties that can be targeted to support adaptive neuronal responses and mental health.

SUMMARY – MENTAL HEALTH DISORDERS AS MILD NEUROLOGICAL OR NEURODEGENERATIVE DISEASE

Neurons and microglia utilize bidirectional interactions to shape form and function of both cell types. In models of stress-induced mental health disorders, concomitant alterations in neuroplasticity and microglia function are reported, reflecting disruptions in neuron–microglia interactions. Based on these characteristics stress-induced microglia activation resembles an immune state termed parainflammation, which is aimed to restore neuronal homeostasis. It is possible that stress exposure elicits modest inflammatory responses that divert microglia from their supportive functions, leading to neuroplasticity deficits underlying anxiety- and depressive-like behaviors. Stress-induced microglia dysregulation may also contribute to neurological complications as clinical evidence shows that individuals with prior history of mental health disorders have increased risk for dementia or neurodegenerative disease (138, 139). Thus, impaired neuron–microglia interactions may link psychological stress exposure and mental health disorders with aging and neurodegenerative disease (15, 140). This is plausible as synapse loss is a common pathophysiological feature observed in depressed individuals and early stages of neurodegenerative diseases (141, 142). Further studies should be conducted to determine if impaired neuron–microglia interactions contribute to the link between, psychological stress, mental health, and neurodegenerative disease. In addition, recent work indicates that sex-dependent differences in microglia function exist and these likely have implications for the pathophysiology of mental health and cognitive disorders as well (143–145). In the end, pharmacological or molecular pathways that engage or promote the adaptive and neurotrophic functions of microglia may provide therapeutic benefits for mental health and neurological disorders.

AUTHOR CONTRIBUTIONS

ESW wrote and edited this manuscript.

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Microglial CD206 Gene Has Potential as a State Marker of Bipolar Disorder

Masahiro Ohgidani¹, Takahiro A. Kato^{1,2*}, Yoshinori Haraguchi³, Toshio Matsushima¹, Yoshito Mizoguchi³, Toru Murakawa-Hirachi³, Noriaki Sagata¹, Akira Monji³ and Shigenobu Kanba¹

¹ Department of Neuropsychiatry, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan, ² Innovation Center for Medical Redox Navigation, Kyushu University, Fukuoka, Japan, ³ Department of Psychiatry, Graduate School of Medical Sciences, Saga University, Saga, Japan

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Björn Tackenberg,
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Björn Spittau,
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*Correspondence:

Takahiro A. Kato
takahiro@npsych.med.kyushu-u.ac.jp

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The pathophysiology of bipolar disorder, especially the underlying mechanisms of the bipolarity between manic and depressive states, has yet to be clarified. Microglia, immune cells in the brain, play important roles in the process of brain inflammation, and recent positron emission tomography studies have indicated microglial overactivation in the brain of patients with bipolar disorder. We have recently developed a technique to induced microglia-like (iMG) cells from peripheral blood (monocytes). We introduce a novel translational approach focusing on bipolar disorder using this iMG technique. We hypothesize that immunological conditional changes in microglia may contribute to the shift between manic and depressive states, and thus we herein analyzed gene profiling patterns of iMG cells from three patients with rapid cycling bipolar disorder during both manic and depressive states, respectively. We revealed that the gene profiling patterns are different between manic and depressive states. The profiling pattern of case 1 showed that M1 microglia is dominant in the manic state compared to the depressive state. However, the patterns of cases 2 and 3 were not consistent with the pattern of case 1. CD206, a mannose receptor known as a typical M2 marker, was significantly downregulated in the manic state among all three patients. This is the first report to indicate the importance of shifting microglial M1/M2 characteristics, especially the CD206 gene expression pattern between depressive and manic states. Further translational studies are needed to dig up the microglial roles in the underlying biological mechanisms of bipolar disorder.

Keywords: bipolar disorder, rapid cycling, microglia, CD206, induced microglia-like (iMG) cells, state marker, M1/M2 polarization, translational research

BIPOLAR DISORDER AND MICROGLIA

The pathophysiology of bipolar disorder has yet to be well understood, while recent studies have indicated abnormal immunological functions may be a contributing factor (1, 2). Microglia, immune cells in the brain, play important roles in the process of brain inflammation, and recent positron emission tomography (PET) studies have shown microglial overactivation in the brain of patients with various psychiatric disorders including bipolar disorder (3–8). Based on the above evidence, microglia has

been highlighted in the study of various psychiatric disorders to understand the underlying biological mechanisms (9–12).

We have recently developed a technique to induced microglia-like (iMG) cells from peripheral blood (13) and are now confirming the utilities of this technique for psychiatric research (14, 15). The underlying mechanisms of the bipolarity between manic and depressive states have yet to be clarified. Immunological conditional changes in microglia may contribute to the manic–depressive shift in bipolar disorder. In the field of immunology, M1/M2 polarization is recognized as a useful marker of macrophages and related cells including microglia. Polarization pattern is well known to distinguish functional

phenotypes: pro-inflammation (M1) and anti-inflammation (M2) (16, 17). Recently, M1/M2 polarization has been highlighted in the understanding of psychiatric disorders (18–20). However, there is no research analyzing M1/M2 polarization of microglia in patients with bipolar disorder. We hypothesize that the expression profile of inflammation-related genes known as M1 (CD45, CD80, HLA-DR, TNF- α , IL-1 β , and IL-23) or M2 markers (CD206, CD209, CD23, BDNF, IL-10, and CCL18) of microglia may shift between manic and depressive states. In order to clarify this hypothesis, we herein analyzed iMG cells from three patients with rapid cycling bipolar disorder during both manic and depressive states, respectively.

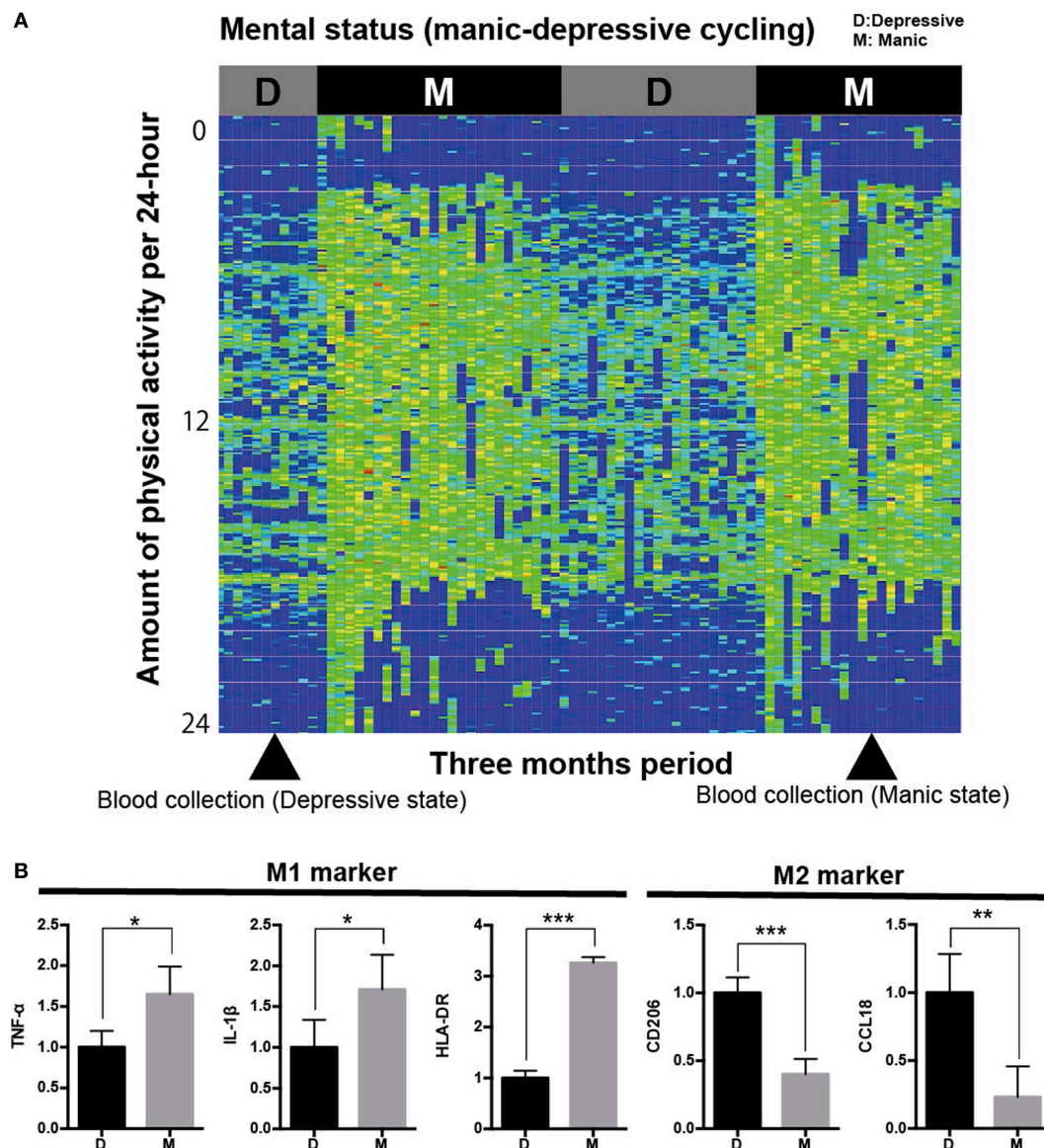


FIGURE 1 | Analysis of iMG cells from a typical case with rapid cycling bipolar disorder. (A) Physical/mental activity of a patient with rapid cycling bipolar disorder for 3 months (case 1). **(B)** Gene profiling pattern of iMG cells from case 1 showed that M1 microglia is dominant in the manic state compared to the depressive state.

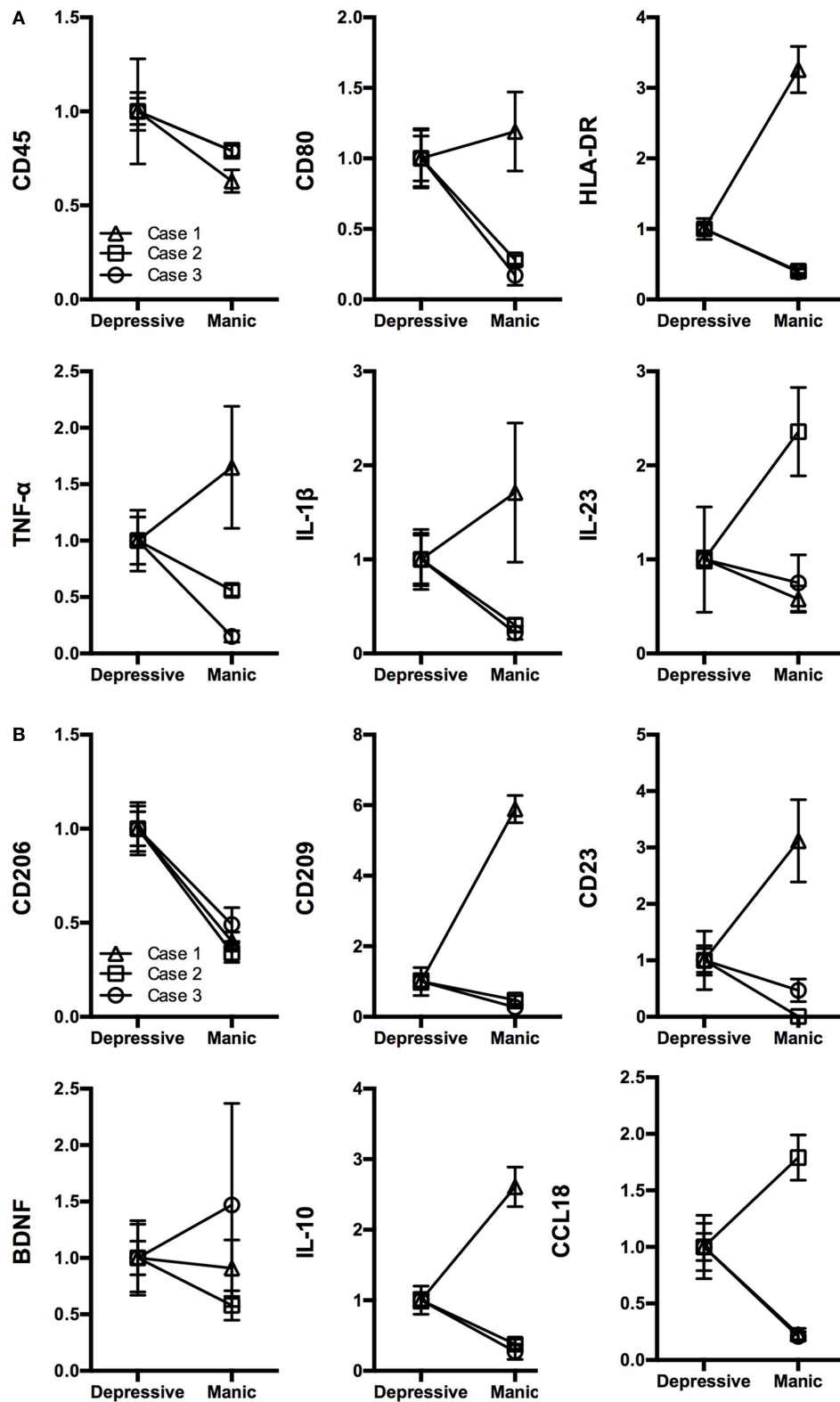


FIGURE 2 | Continued

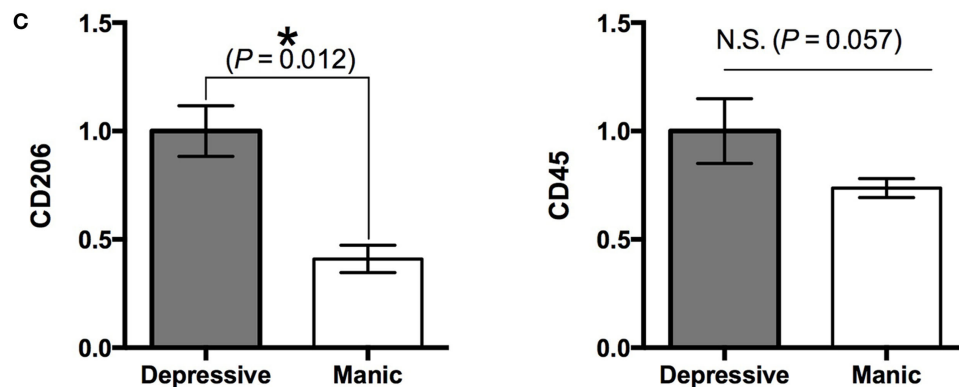


FIGURE 2 | Gene profiling pattern of iMG cells between depressive and manic states among three patients. (A) M1 markers, (B) M2 markers, and (C) statistical analysis of CD206 and CD45 among the three patients (mean value \pm SD).

M1/M2 MICROGLIA AND BIPOLAR DISORDER

Patients' demographic data are shown in Table S1 in Supplementary Material. We produced iMG cells of each patient from both manic and depressive states and compared the gene expression profiles between both states. Relative gene expression (normalized by depressive state) of M1 and M2 markers are shown in **Figures 1** and **2A,B**, respectively.

We revealed that the gene profiling patterns of iMG cells are different between manic and depressive states. The profiling pattern of case 1 showed that M1 microglia is dominant in the manic state compared to the depressive state (**Figure 1B**). However, the patterns of cases 2 and 3 were not consistent with the pattern of case 1 (**Figures 2A,B**).

For M1 markers, CD45 was downregulated in the manic state among all three patients. Other M1 markers such as TNF- α , IL-1 β , IL-23, CD80, and HLA-DR shifted differently among the patients (**Figure 2A**). For M2 markers, CD206 was downregulated in the manic state among all three patients. Other M2 markers such as BDNF, IL-10, CCL18, CD23, and CD209 shifted differently among the patients (**Figure 2B**). Thus, we performed statistical analysis [Student's *t*-test (two-tailed)] among the three patients. As shown in **Figure 2C**, CD206 was significantly downregulated in the manic state ($p = 0.012$). On the other hand, CD45 showed no significant difference between manic and depressive states ($p = 0.057$).

CD206 AND MICROGLIA

In the present study, we have shown that downregulation of the CD206 gene of iMG cells in the manic state was consistent across all three patients with bipolar disorder. CD206, known as a mannose receptor, is a 175-kDa transmembrane protein, mostly expressed by macrophages, dendritic cells, and endothelial cells. This receptor selectively and efficiently captures mannosylated ligands such as microbial antigen (21). In the brain, CD206 is also expressed

in microglia (22, 23) and astrocytes (24, 25). CD206 is widely recognized as a typical M2 microglial marker (23, 26, 27). CD206 has some important cellular functions especially in pinocytosis and phagocytosis on microglia (22, 25, 28–30). Therefore, CD206 may be critical as the first step in the recognition and capture of pathogens in the brain (25). To date, there are no studies focusing on microglial CD206 in psychiatric disorders including bipolar disorder. In the present study, CD206 was downregulated in the manic state. This finding might suggest that the manic state of microglia is more vulnerable to the pathogen and/or insoluble matter compared to the depressive state. Based on the present results, M2 microglia may be dominant in the depressive state in patients with bipolar disorder, especially rapid cycling patients. Further translational investigations should be conducted to clarify our hypothesis.

LIMITATION AND FUTURE PERSPECTIVES

One major limitation of the present study is that all three patients were on medication. Given that it took 2 weeks to induce iMG cells, the influence of medication is assumed to be minimal. Additional studies are needed in medication-free patients. On the other hand, recent PET studies have suggested microglial overactivation in patients with bipolar disorder (7, 8). Thus, human PET studies should be conducted in patients with bipolar disorder during both manic–depressive states. However, even the most advanced brain imaging techniques cannot analyze M1/M2 polarization in the human brain, and thus we believe that the iMG technique has an advantage for such analyses in translational research.

A recent study has revealed that the origin of brain microglia is primitive macrophages migrated from the yolk sac before embryonic day 8 (31). One of the limitations in the present study is that iMG cells are not actual microglial cells in the brain. However, we believe that our iMG cells are surrogate cells, which can represent some of the characteristics of brain microglial cells (13). Further comparison studies are needed to investigate the similarity and

differences between brain microglial cells and iMG cells in more detail.

The life span of intravascular blood monocytes is only a few days long (32). Thus, we believe that the iMG cells from peripheral blood monocytes are useful not only as a trait marker but also as a state marker in order to assess a variety of mental states. However, an inherent limitation of our iMG analysis is the time delay between the date of blood collection and the date of analysis of iMG cells (after 14 days) due to the necessity of 14-day induction from blood monocytes. Further investigations should be conducted to clarify the impact of time delays in analysis of iMG cells.

The underlying biological mechanism shifting the expression patterns of iMG cells between depressive and manic states has not been clarified at present, while some internal and/or external factors are suggested to contribute to this shifting mechanism. Recent immunological studies have suggested that immune cell activities, including microglia, are modulated by the circadian clock system (33), methylation (34), and/or external stress (35), which may contribute to the activation patterns of microglia during clinical courses of bipolar disorder.

A previous study has shown that peripheral blood mononuclear cells from patients with rapid cycling bipolar disorder presented a different pattern of gene expression between manic and depressive states (36). In addition to the present study, this report also supports the premise that the cellular phenotype of microglia including M1/M2 state is different between depressive and manic states. Further studies are required to determine the clinical importance of these pilot findings.

CONCLUSION

We introduced a novel translational approach focusing on bipolar disorder using the iMG technique. To our knowledge, this is the first report to indicate the importance of shifting microglial CD206 gene expression between depressive and manic states. This study is the first step toward understanding the contribution of microglia to the pathogenesis of bipolar disorders. Further studies are needed to dig up the microglial roles in bipolar disorder.

ETHICS STATEMENT

The present study was conducted in accordance with the Declaration of Helsinki and was approved by the Ethics

Committee of the Graduate School of Medical Sciences, Kyushu University.

AUTHOR CONTRIBUTIONS

All the authors contributed substantially to the scientific process leading up to the writing of the present manuscript. TK: the principal investigator of the present research; MO: the first author created the conception and design of the project and wrote the protocol. YH, TM, YM, TM-H, and AM: performed the clinical recruitment. MO, TK, YH, TM, YM, NS, and TM-H: performed the experiments and data analyses/interpretation. MO: wrote the first draft of the manuscript. TK, AM, YM, and SK: made critical revisions of the manuscript. All the authors approved this submission in its current form.

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Environmental Toxicants-Induced Immune Responses in the Olfactory Mucosa

Fumiaki Imamura* and Sanae Hasegawa-Ishii

Department of Pharmacology, Penn State College of Medicine, Hershey, PA, USA

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*Correspondence:

Fumiaki Imamura
fimamura@hmc.psu.edu

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Olfactory sensory neurons (OSNs) are the receptor cells for the sense of smell. Although cell bodies are located in the olfactory mucosa (OM) of the nasal cavity, OSN axons directly project to the olfactory bulb (OB) that is a component of the central nervous system (CNS). Because of this direct and short connection from this peripheral tissue to the CNS, the olfactory system has attracted attention as a port-of-entry for environmental toxicants that may cause neurological dysfunction. Selected viruses can enter the OB via the OM and directly affect the CNS. On the other hand, environmental toxicants may induce inflammatory responses in the OM, including infiltration of immune cells and production of inflammatory cytokines. In addition, these inflammatory responses cause the loss of OSNs that are then replaced with newly generated OSNs that re-connect to the OB after inflammation has subsided. It is now known that immune cells and cytokines in the OM play important roles in both degeneration and regeneration of OSNs. Thus, the olfactory system is a unique neuroimmune interface where interaction between nervous and immune systems in the periphery significantly affects the structure, neuronal circuitry, and immunological status of the CNS. The mechanisms by which immune cells regulate OSN loss and the generation of new OSNs are, however, largely unknown. To help develop a better understanding of the mechanisms involved, we have provided a review of key research that has investigated how the immune response in the OM affects the pathophysiology of OSNs.

Keywords: olfactory epithelium, inflammation, immune system, intranasal administration, olfactory vector hypothesis, olfactory dysfunction, neurodegenerative disease

INTRODUCTION

We are continuously exposed to a variety of potentially harmful environmental agents, such as bacteria, viruses, mold, dust, pollen, and environmental chemicals. Environmental agents entering the nasal cavity may become allergens, causing inflammation in the olfactory mucosa (OM) (olfactory inflammation), and leading to allergic rhinitis and infectious sinusitis (1). The symptoms are usually associated with hyposmia or anosmia (2, 3). Olfactory loss in rhinitis/sinusitis is attributable primarily to blockade of airflow to the olfactory sensory neurons (OSNs) that receive odorous molecules, but damage to the OM is also considered as a possible cause (2, 4–6). In fact, multiple studies have shown that olfactory inflammation causes the loss of OSNs (7–14).

Epidemiological studies have associated exposure to environment toxicants with the incidence of neurodegenerative diseases, including Alzheimer's and Parkinson's diseases (15, 16). Since olfactory dysfunction is a common prodromal symptom of these diseases, and because xenobiotics administered into the nasal cavity are often found in the brain, the nasopharynx has attracted

attention as a port-of-entry for environmental agents that cause neurological disease (the olfactory vector hypothesis) (17–19). To date, a variety of neurotoxicants have been directly administered to the naris of model animals to study their transport to the brain and the resultant neurodegenerative effects in the central nervous system (CNS). Results of these types of studies have now been summarized in a number of reviews (17, 20–22).

Neuroinflammation is a hallmark of neurodegenerative diseases (23–26). Although knowledge of the cause of neuroinflammation is still limited, olfactory inflammation has been proposed as one of the major mechanisms (27, 28). Interestingly, allergic rhinitis is associated with development of Parkinson's disease later in life (29). We, therefore, believe that a better understanding of the olfactory immune system will advance our knowledge of the pathogenesis and progression of neurological disease. To date, there are many reports showing that artificially induced olfactory inflammation can cause immune responses and damage to the OM (7–14). Conversely, new OSNs are generated in the OM throughout life, which may help in the repair of damaged tissue (30, 31). It also has been reported that immune cells in the OM regulate the depletion of old OSNs and generation of new OSNs. This review summarizes the roles of immune cells in the inflammatory response, tissue damage, and regeneration of the OM with a focus on model systems, primarily the OM of murine species.

Structure of the Olfactory Mucosa

The OM is located in the upper region of the nasal cavity, and is made up of the olfactory epithelium (OE) and the underlying lamina propria (**Figure 1**). The surface of the OE is covered with a mucus layer where inhaled odorant molecules can be trapped, which then bind to odorant receptors expressed on the cilia of the OSNs whose cell bodies are located in the OE. Unlike other receptor cells, OSNs project directly to the olfactory bulb (OB), the first relay station of olfactory information in the CNS, through the cribriform plate. Sustentacular cells line the apical surface of the OE, and provide trophic, metabolic, and mechanical support for OSNs. At the basal surface of the OE, there are two types of basal cells (horizontal and globose basal cells) that give rise to new OSNs and sustentacular cells during lifetime of the organism.

The lamina propria is a layer of connective tissue through which OSN axons pass. OSN axons, although unmyelinated, are gathered into bundles (olfactory nerve) that are wrapped with olfactory ensheathing cells (OECs). OECs are specialized glial cells that are resident in the olfactory system. The lamina propria also contains Bowman's glands and vascular elements. Bowman's glands produce mucus and secrete it to the mucus layer *via* the OE duct.

Response of Olfactory Mucosa to Intranasal Administration of Environmental Agents

Inhalation of harmful environmental agents often damages the OM. Here, we focus on the damages associated with inflammatory responses within the nasal cavity (32). Several

animal models of human chronic rhinosinusitis have been developed by inoculating bacteria or fungus extract into the mouse nostril (10, 33, 34). These mouse models have shown inflammatory responses in the nasal cavity, as well as general pathology of the OE that includes mast cell and eosinophilic infiltration into the respiratory epithelium, with increased depth of lamina propria. In addition, olfactory inflammation can be caused by a single compound derived from microbial pathogens, such as polyinosinic:polycytidylic acid [Poly(I:C)] (14), lipopolysaccharide (LPS) (9), satratoxin G (SG), and roridin A (RA) (7, 8, 11, 13). Poly(I:C) is a synthetic analog of viral double-stranded RNA, and is recognized by Toll-like receptor 3 (TLR3) (35), whereas LPS is an endotoxin found in the outer membrane of Gram-negative bacteria that activates another type of Toll-like receptor, TLR4 (36, 37). When injected intraperitoneally, LPS caused systemic inflammation that also changes the level of inflammatory cytokines in the brain (38). SG and RA are macrocyclic trichothecene mycotoxins produced by fungi such as *Stachybotrys chartarum*, the “black mold” (39). Immunohistochemical analyses using TLR3 and TLR4 antibodies indicated that sustentacular cells and OECs may be the first target cells of Poly(I:C) and LPS in the OM, respectively (14, 40). Besides activating different receptors, therefore different types of cells, intranasal inoculation of each of these agents causes an inflammatory response and damage to the OM of rodents. It is useful to review what is known about intranasal inoculation of Poly(I:C), LPS, SG, and RA and their effects on olfactory tissues.

Inflammatory Response

Infiltration of neutrophils expressing Ly-6G/-6C into the OM occurs 1 day after intranasal inoculation of Poly(I:C), SG, or RA (7, 8, 14). Kanaya et al. confirmed that Poly(I:C) caused the infiltration of macrophages (F4/80+) and T-lymphocytes (CD3+) (14). In contrast to the situation with neutrophils, which completely disappeared within 6 days, significantly higher numbers of macrophages and T-lymphocytes were observed in the OM as long as 21 days after the last Poly(I:C) inoculation. In addition, in the OM, Poly(I:C), SG, or RA caused upregulation of mRNAs encoding inflammatory cytokines, including IL-1 α , IL-1 β , IL-6, TNF- α , and MIP-2.

Damage of the Olfactory Mucosa

Intranasal inoculation of environmental agent-derived components also damaged the OM and led to apoptosis of OSNs and decreased thickness of the OE (7–9, 14). When Poly(I:C) was inoculated into mouse nostril once a day for 3 days, the number of apoptotic cells was significantly increased and the number of OSNs was decreased in first 3 days. When examined 9 days after the first inoculation (i.e., 6 days after the last inoculation), few apoptotic cells were observed in the OE, but the number of OSNs was less than that observed 3 days post inoculation.

The mechanisms underlying OSN loss associated with olfactory inflammation are currently not well understood. Inflammatory responses seem to play a critical role for death of OSNs. During inflammation, neutrophils and macrophages

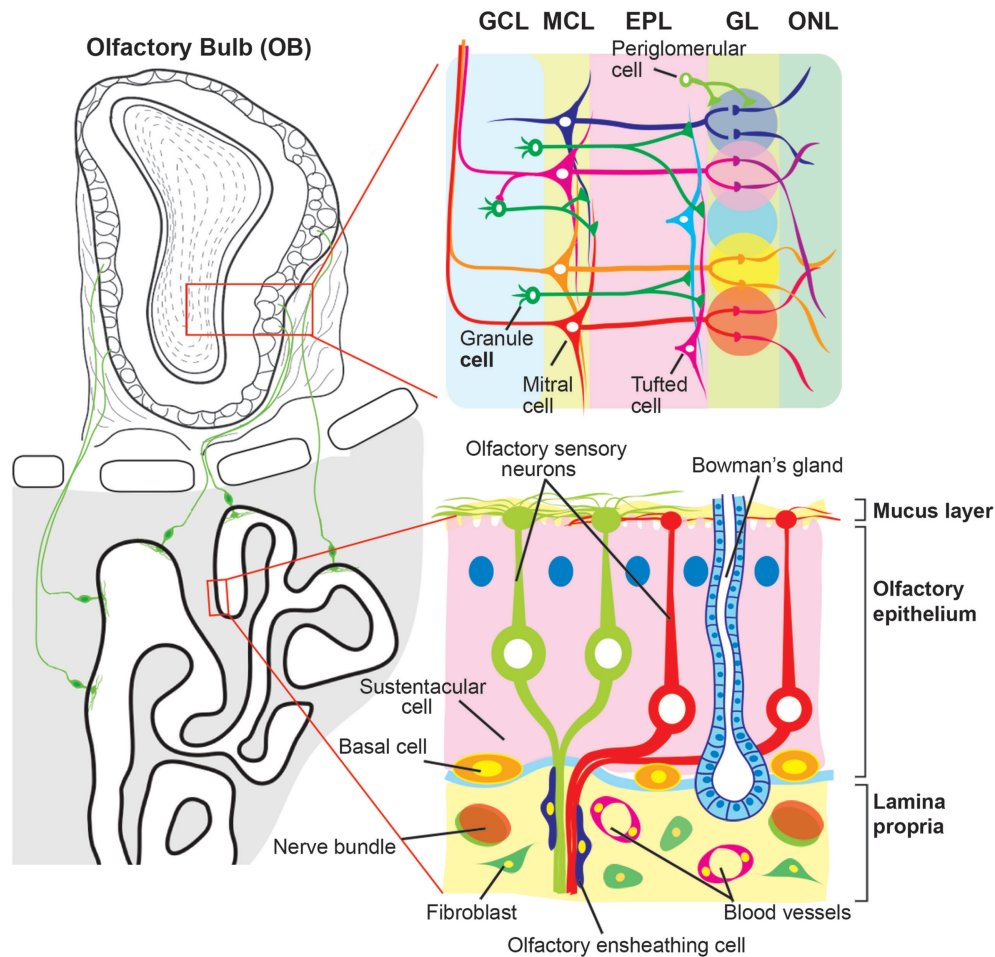


FIGURE 1 | Schematic diagram of the olfactory mucosa and olfactory bulb. The olfactory mucosa is composed of the olfactory epithelium (OE) and the lamina propria. Three types of cells are found in the OE, olfactory sensory neurons (OSNs), sustentacular cells, and basal cells. The dendrites of OSNs project toward the mucus layer where they protrude the cilia expressing odorant receptors. Sustentacular and basal cells are localized at the apical and basal regions of the OE, respectively. The lamina propria is a layer of connective tissue, which lies beneath the OE, and contains fibroblast, blood vessels, and Bowman's gland. OSN axons are fasciculated into bundles that are wrapped with olfactory ensheathing cells and target the olfactory bulb (OB) by passing through the lamina propria and cribriform plate. The OB is divided into multiple layers. Each OSN axon runs the surface of the OB, olfactory nerve layer (ONL), and projects to a glomerulus in the glomerular layer (GL). There, the OSN axons synapse with primary dendrites of projection neurons, mitral and tufted cells, and onto populations of interneurons, periglomerular cells. The secondary dendrites of mitral/tufted cells make dendrodendritic synapses with another population of interneurons, granule cells, in the external plexiform layer (EPL). Beneath the EPL, the OB has the mitral cell layer (MCL) and granule cell layer (GCL) where the cell bodies of mitral cells and granule cells are located, respectively.

secrete elastases, proteases known to break down bacterial membrane proteins (41). Intranasal inoculation of neutrophil elastase caused the loss of OSNs in the OE (14). In contrast, Poly(I:C)-induced damage of the OM was blocked by prior intraperitoneal injection of the neutrophil elastase inhibitor, Silevestat (14). In addition, inflammatory cytokines are also involved in inflammation-induced OSN death. Lane and colleagues created a transgenic, induced olfactory inflammation (IOI) mouse model, in which expression of TNF- α in sustentacular cells was induced with doxycycline as a chronic rhinitis model (12). Using this transgenic mouse, Lane et al. showed that induction of TNF- α expression caused marked reduction of OE thickness and loss of OSNs, whereas the sustentacular cells were unaffected. Concurrent treatment with prednisolone (to inhibit downstream

inflammatory responses) prevented OSN loss, therefore suggesting that TNF- α does not directly cause OSN apoptosis (42).

The damage to the OM exacerbates the impact caused by exposure to environmental toxicants. While *Staphylococcus aureus* is an indigenous microbe found in the nose and usually remains in the lumen after intranasal administration, bacteria can still penetrate the OE and cause an inflammatory response when inoculated intranasally after first damaging the OM with Triton X-100 or zinc sulfate (43). In addition, tissue damage coupled with an inflammatory response of the OM induced by RA was exacerbated by co-exposure to LPS (8). Although mRNAs encoding inflammatory cytokines were marginally induced by RA alone, co-exposure to both RA and LPS dramatically elevated expression of these genes.

Regeneration of OE

Basal cells of the OE give rise to new OSNs and sustentacular cells throughout life (44, 45). Damage of basal cells caused by methyl bromide gas, however, led to the eventual loss of OSNs and inhibited the reconstruction of OE (46). In contrast, these basal cells seem to be less affected by intranasal inoculation of Poly(I:C), LPS, or mycotoxins; apoptosis was restricted to OSNs whose cell bodies reside in the middle layer of the OE, below the apical row of supporting cell nuclei and above the basal cell nuclei (7, 8, 14). Cells expressing Ki67, a proliferating basal cell marker, were increased in number and distributed in all layers of the OE 6 days after the last Poly(I:C) inoculation (14). The OE thickness and the OSN number were almost completely recovered by 21 days post-Poly(I:C) exposure. However, the recovery of OSNs was incomplete (40–50%), even at 21–28 days after the last inoculation of SG (7, 11).

Immune cells and OECs in the OM also regulate many aspects of degeneration/regeneration of OSNs. The resident macrophages play a key role in the removal of cell debris and stimulation of basal cells to proliferate (47). OEC is known to be a specialized type of glia that wraps OSN axons, as well as serves as a major phagocytic cell type (48, 49). Bulbectomy or olfactory nerve transection causes apoptotic death of OSNs by severing the axons and stimulates the generation of new OSNs (50–53). Bulbectomy activates the proliferation of OECs in lamina propria (54) and the infiltration of macrophages into the OM (55). In addition to the phagocytosis of apoptotic cellular debris, infiltrated macrophages secrete a variety of inflammatory cytokines and chemokines, such as LIF, IL-6, MCP-1, and MIP-1 α (52, 56, 57). It has been proposed that MCP-1 and MIP-1 α play key roles in recruitment of additional macrophages to the OM and that LIF stimulates globose basal cells expressing LIF receptor (LIFR) (52, 57, 58). Activation of LIFR subsequently induces iNOS expression that in turn stimulates proliferation of neural precursor cells (59). An increase in iNOS level was also observed in OECs after bacterial challenge to the compromised OM (60).

DISCUSSION

This review has summarized olfactory inflammation caused by intranasal inoculation of Poly(I:C), LPS, and mycotoxin. Although the receptors and signaling pathways activated by these agents are not identical, they induce similar effects on the OM, including infiltration of immune cells, upregulation of inflammatory cytokines, and loss of OSNs. It appears that damaged and lost OSNs can be replaced with new OSNs since olfactory inflammation has minimal effect on the basal cells. It is not clear, however, whether basal cells are, in fact, affected by olfactory inflammation. The inoculation of toxicants into the LOI-transgenic mouse showed that TNF- α -induced inflammation lasted for 6 weeks and compromised the regeneration of OSNs, although the effect was not permanent, suggesting that TNF- α suppresses the proliferative activity of basal cells (12, 61). In contrast, intraperitoneal injection of the herbicide 2,6-dichlorobenzonitrile induced inflammation-like pathological changes in OE and depleted the horizontal basal cells, resulting in permanent loss of OSNs (62). A critical next step is to elucidate the molecular mechanisms

underlying the specific loss of OSNs and the resistance of basal and sustentacular cells to olfactory inflammation. Since variety of immune cells are involved in inflammatory responses, detailed researches on types of immune cells activated and infiltrated in the OM during olfactory inflammation are required to elucidate the mechanisms.

It is also known that zinc sulfide and hydrogen sulfide administered into the nasal cavity induces the loss of OSNs (63–67), and anosmia induced by intranasal zinc has been suggested to occur in humans (68, 69). The immune response caused by exposure of the nasal cavity to toxic gases and metals is not well understood, but the regions of the OM affected by hydrogen sulfide inhalation is different from the regions affected by either intranasal Poly(I:C), LPS, or by mycotoxin inoculation. The OE can be subdivided into several zones based on the expression patterns of specific molecules (including olfactory receptors), and the dorsal medial meatus largely overlaps with zone 1 (aka dorsal zone) (70). Inhalation of hydrogen sulfide provoked necrotizing lesions of the OSNs predominantly localized in the zone 1 (64, 65), whereas the OE lining the dorsal medial meatus was not affected by the intranasal inoculation of Poly(I:C), SG, or RA solution (7, 11, 14). The difference in susceptible portions in the OE may be attributed to the different flows of liquid and gas in the nasal cavity (71). Alternatively, molecules exclusively expressed by OSNs in zone 1 (e.g., NQO1, O-MACS, and Dvl-1) or in zone 2–4 (e.g., OCAM and Foxg1) may determine the susceptibility of OSNs to the environmental agents (72–76). Understanding the similarities and difference in immune responses to different environmental agents will help us to evaluate the risks to the CNS.

According to the olfactory vector hypothesis, some neurological disorders are caused or accelerated by agents entering the OB *via* the OM (17). Of interest, it was shown that either intranasally administered influenza virus, LPS, or MPTP (a synthetic neurotoxicant) caused selective decreases of dopamine neurons in the substantia nigra of mice (21, 27, 77–79). The route from the OM to the substantia nigra, however, remains to be elucidated. The transport of viruses, bacteria, and metals from the nasal cavity to the OB has been reviewed by others (17, 20–22). Interestingly, Nipah virus propagates anterogradely in the hamster CNS *via* the olfactory pathway beginning in the OB (80). The agents entering the OB may spread further in the brain to cause neurological disorders. It is suggested that inflammatory responses can spread in the CNS both anterogradely or retrogradely *via* axonal projections (81). For instance, corneal inflammation induced by instillation of benzalkonium chloride damages primary sensory neurons in the trigeminal ganglion, leading to the activation of second-order neurons and glial cells in the brain stem and to the production of pro-inflammatory cytokines (82). Therefore, the inflammatory response may propagate in the brain from the primary olfactory tissue. Although this review focused on the effects on the OM, olfactory inflammation was also associated with atrophy of the OB; upregulation of the mRNA levels of inflammatory cytokines; infiltration of neutrophils; and/or activation of astrocytes and microglia (7, 8, 11, 43). These changes clearly should affect the OB neurons. Furthermore, intranasal LPS injection caused upregulation of TLR2 signals in the OB, which spread to other parts of the brain within 24 h (83). Further studies of neuroinflammation

and damage in other brain regions will provide us with novel insights into the olfactory vector hypothesis and the pathogenesis of neurological disorders.

CONCLUSION

The olfactory system is a unique site where the peripheral nervous system and CNS are in close proximity. Since the OM is bathed in a sustained exposure of environmental agents that may cause inflammatory responses, the health of the CNS is likely to be heavily influenced by the immune status of the olfactory system. Big challenges in future are (1) to determine whether olfactory inflammation contribute to pathogenesis of neurodegenerative diseases; and (2) to determine whether olfactory inflammation

sequentially affect the immune status of the CNS *via* the olfactory pathways in the brain.

AUTHOR CONTRIBUTIONS

FI and SH-I searched and reviewed previous works and wrote this article.

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Neuroimmune Interface in the Comorbidity between Alcohol Use Disorder and Major Depression

Sudan Prasad Neupane^{1,2*}

¹ Norwegian National Advisory Unit on Concurrent Substance Abuse and Mental Health Disorders, Innlandet Hospital Trust, Brumunddal, Norway, ² Norwegian Centre for Addiction Research (SERAF), University of Oslo, Oslo, Norway

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Edited by:

Ilhassane Zouikr,
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University Hospital Essen, Germany

*Correspondence:

Sudan Prasad Neupane
sudan.prasad.neupane@
sykehuset-innlandet.no,
thelonelyplanet@gmail.com

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Bidirectional communication links operate between the brain and the body. Afferent immune-to-brain signals are capable of inducing changes in mood and behavior. Chronic heavy alcohol drinking, typical of alcohol use disorder (AUD), is one such factor that provokes an immune response in the periphery that, by means of circulatory cytokines and other neuroimmune mediators, ultimately causes alterations in the brain function. Alcohol can also directly impact the immune functions of microglia, the resident immune cells of the central nervous system (CNS). Several lines of research have established the contribution of specific inflammatory mediators in the development and progression of depressive illness. Much of the available evidence in this field stems from cross-sectional data on the immune interactions between isolated AUD and major depression (MD). Given their heterogeneity as disease entities with overlapping symptoms and shared neuroimmune correlates, it is no surprise that systemic and CNS inflammation could be a critical determinant of the frequent comorbidity between AUD and MD. This review presents a summary and analysis of the extant literature on neuroimmune interface in the AUD–MD comorbidity.

Keywords: alcohol use disorder, depression, comorbidity, neuroimmune interface, neuroinflammation, alcohol drinking, cytokines

INTRODUCTION

Alcohol consumption is responsible for 5.9% of global annual deaths and 5.1% of the global disease burden (1). Unipolar major depression (MD) was the second leading cause of years lived with disability worldwide, accounting for 8% of all global years lived with disabilities in 2013 (2). Together, alcohol use disorder (AUD) and MD disorders account for a half of the global disease burden attributable to mental and substance use disorders (3). An unequivocally high comorbidity exists between AUD and MD, with a lifetime comorbidity rate of 20.5% (4). About 30% of individuals with MD report lifetime AUD (5). Conversely, depressive symptoms are common in AUD to the extent that well over a third of AUD patients satisfies diagnostic criteria for MD at some point during their drinking career (6, 7). Compared to isolated disorder, patients with AUD–MD comorbidity carry higher risk of relapse to alcohol dependence, treatment dropout, suicide attempt, and poorer effect of antidepressant medication and have lower global functioning and less life satisfaction (4, 8, 9). Attempts to disentangle causal pathways between depression and AUD have resulted in the wider acceptance of bidirectional causality, with an estimate suggesting one disorder doubles the risk for the other (10). However, the mechanisms of such causality and the interfaces at which they interact remain unclear.

A colloquial understanding of the brain–body interaction is that the brain subjugates the body and pathogenic penetration of the blood–brain barrier is the only route by which bodily immune

insults can reach the brain tissue. This view has changed with the demonstration of immune signals in the form of inflammatory cytokines that access the brain *via* afferent vagal fibers (11), by directly crossing leaky regions in the blood–brain barrier (e.g., area postrema), through cytokine-specific active transport molecules and through secondary messenger molecules within the CNS endothelia (12). Microglia and astrocytes can in turn accentuate CNS cytokine load. These cytokines and the relayed signals in the brain interact with various neurotransmitter systems as well as the hypothalamic–pituitary–adrenal (HPA) axis, the primary hormonal response system to stress (13). Furthermore, co-stimulatory signals that allow mast cells to interact with the immune cells and influence the integrity of the blood–brain barrier are important mediators of the cross talk between the peripheral and the central neuroimmune signaling (14). Thus, immune inflammatory signals in the brain are key to the translation of psychological and biological stressors into behavioral outcomes.

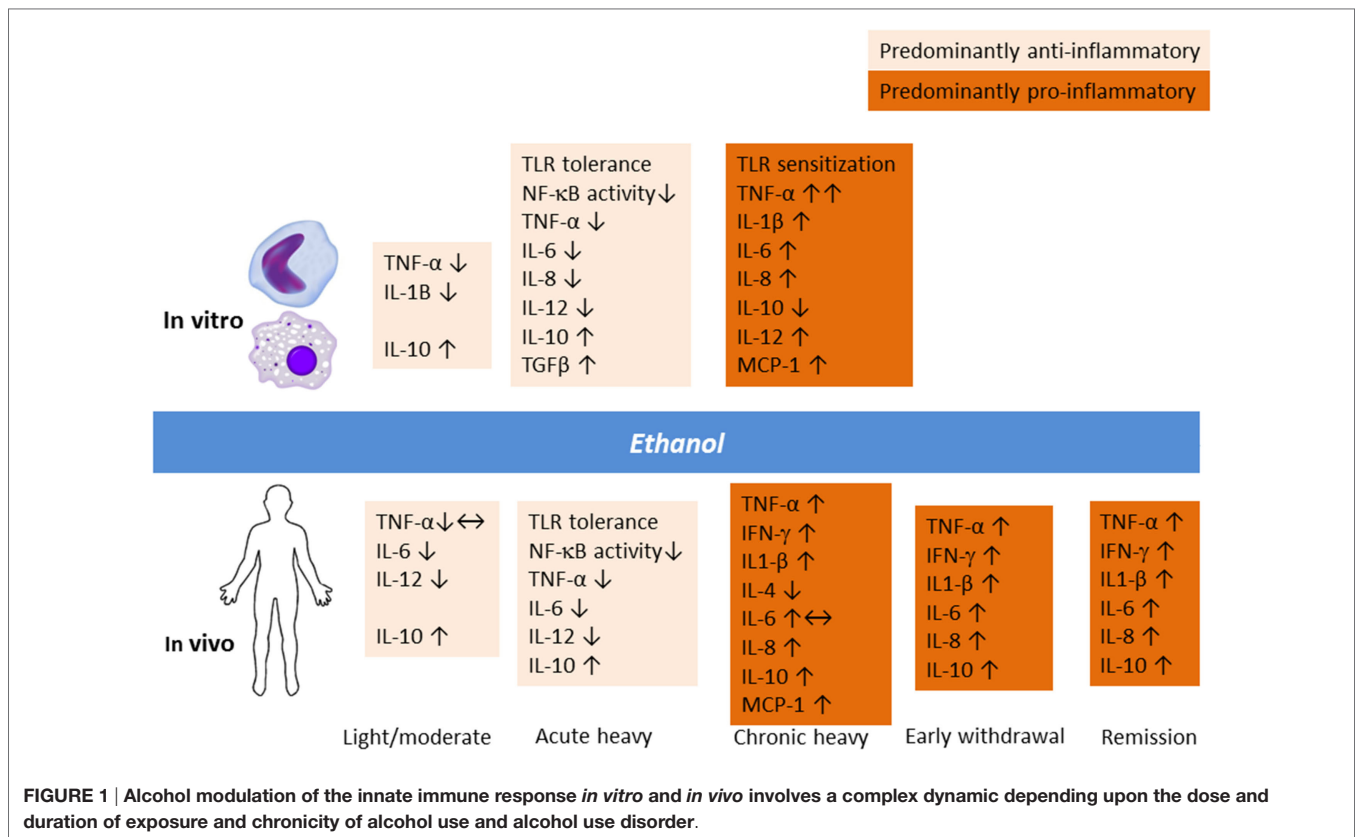
Several lines of research show both AUD and MD are, as isolated disorders, associated with various changes in immune function. There is, however, a paucity of knowledge on the role of neuroimmune function in the development and progression of comorbid AUD and MD. As an example, a binge pattern of drinking is particularly depressogenic (10), but the exact underlying neurobiological mechanism for this “alcoholic depression” awaits elucidation. The available evidence indicates that allostatic changes in the neuroimmune functioning could have significant impact on the development, progression, and outcome of AUD–MD comorbidity, and promising neuroimmune targets are being identified to address these issues. Several caveats remain before these developments in psychoneuroimmunology of comorbid psychiatric disorders could be capitalized.

AUD AND IMMUNITY

Alcohol is a potent modulator of the immune system and alters the expression of inflammatory mediators in the periphery as well as in the CNS. A well-described mechanistic explanation for this is that heavy alcohol consumption activates toll-like receptor (TLR) systems, including the TLR2 and TLR4 (15), through the danger-associated molecular pattern signaling, which renders the gut wall “leaky” then enabling the translocation of microbial products such as lipopolysaccharides (LPS) into circulation. This effect has been confirmed both in binge drinking (16) and chronic heavy drinking among humans (17, 18) and more widely in animal models (19, 20). The leaked LPS potentiates alcohol-induced liver inflammation and stimulates immune cells such as monocytes, macrophages, T lymphocytes, and dendritic cells to cause the release of pro-inflammatory cytokines, including interleukin (IL)-1 β , IL-6, and tumor necrosis factor- α (TNF- α) (21). Peripherally produced cytokines and chemokines [e.g., monocyte chemoattractant protein-1 (MCP-1)] and/or their signals eventually relay to multiple brain regions, where they further activate brain microglia and astrocytes to produce CNS cytokines. The cytokine production in the brain is again dependent on TLR4 signaling and is propagated along the mitogen-activated protein kinase and NF- κ B pathways. It appears

that alcohol-induced cytokine upregulation follows the pattern of LPS but with less intensity. Within an hour of an intoxicating dose (5 g/kg) of ethanol, IL-10 levels were already significantly increased in rat hippocampus (22). Qin and colleagues demonstrated that comparable doses of ethanol in binge and chronic alcohol drinking paradigm in mice could induce IL-1 β , TNF- α , and MCP-1 production in the liver, plasma, and brain tissues (23). In the liver and other peripheral organs, cytokine upregulation upon LPS or alcohol resolves within days to weeks. Remarkably though, brain immune activation induced by ethanol, or by LPS upon sensitization with ethanol, persisted for many months (23, 24). Using postmortem brain samples, the same group discovered that MCP-1 concentrations were increased in the ventral tegmental area, substantia nigra, hippocampus, and amygdala of alcoholic brains compared to the MCP-1 concentrations in those brain areas of moderate drinking controls (25). Since these areas are relevant to reward, emotion, and behavioral functions, MCP-1 is potentially involved in the neurodegenerative pathologies of alcohol. It is at this juncture that alcohol-induced neuroinflammation becomes clinically relevant because persistent neuroinflammation clearly precipitates cognitive and behavioral responses (26). It has recently been proposed that neuroimmune signaling is an important contributor to the development and maintenance of alcohol dependence (27). Thus, the enduring nature of the neuroimmune induction in the brain resonates with the chronicity of alcohol addiction and might represent a mechanism contributing to the development of closely comorbid conditions of alcohol dependence, such as depression (23, 24).

Alcohol modulation of the immune system involves a complex dynamic dependent on the dose and duration of exposure and chronicity of AUD (**Figure 1**). Acute heavy alcohol consumption (e.g., ≥ 3 g/kg), even in a single dose, inhibits inflammatory cell activation (28–30). Upon LPS challenge, alcohol-primed mice suppressed lung TNF- α activity, TNF-Rp55 mRNA expression, and soluble TNF-Rp55 levels (31). Ethanol suppressed LPS-induced expression of IL-1, IL-6, and their receptors while significantly upregulating IL-10 levels. In fact, acute ethanol blunted LPS-induced TNF- α secretion by 40%. This immune suppressing effect of alcohol drinking has long been appreciated. The exact molecular mechanism for the opposing immune effects of acute and chronic alcohol remains unclear. However, alcohol-induced tolerance and sensitization of TLRs depending on the length of exposure to alcohol may play a role. Through a series of experiments on human monocytes stimulated with LPS and on animal binge drinking models, Szabo and colleagues demonstrated that acute alcohol induces TLR4/LPS tolerance through activation of a nuclear protein Bcl-3, which interacts with the p50 subunit of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) (32). The Bcl-3–NF- κ B p50 interaction results in the suppression of transcription of NF- κ B-regulated genes, including that of pro-inflammatory cytokines (33). Furthermore, chronic alcohol switched the anti-inflammatory response to a pro-inflammatory response by human monocytic sensitization to LPS through decreased expression of interleukin-1 receptor-associated kinase-M, a negative regulator of TLR signaling, and subsequent activation of NF- κ B, an effect opposite to acute alcohol (34).



Animal studies have demonstrated that an anti-inflammatory effect of acute binge drinking ensues already in the first hour and lasts beyond ethanol elimination from the body (22, 31). The same finding was replicated in humans 13 h after intake of 1.5 g/kg of alcohol (35). The experiment showed that stimulated peripheral blood mononuclear cell production of IL-10 and IL-12 as well as IFN- γ was increased upon early withdrawal. Thus, the continuum of heavy drinking, withdrawal, craving, and relapse to alcohol use potentially involves immune inflammatory signaling, an area that deserves further investigations. Recently, a study of rat models of acute alcohol intoxication suggested that the expression of inflammatory cytokines is elevated during the withdrawal phase, but changes in the central nervous system appeared to be site dependent (36). In particular, IL-6 levels were increased in multiple brain regions following alcohol exposure and lasted for up to 18 h. Thus, acute heavy drinking favors apoptotic and anti-inflammatory changes (37, 38), whereas chronic heavy drinking is known to induce monocytic TNF- α production as well as T- and B-cell activation (38, 39).

Clearly, the neuroimmune and endocrine modulatory function of alcohol varies depending on whether the individual is a social drinker or has a severe AUD (**Figure 1**). Gonzalez-Quintela et al. (40) reported that, among Spanish adult men and women, light-to-moderate alcohol drinking was not associated with altered levels of TNF- α . In clinical AUD populations, however, levels of cytokines are typically increased compared to non-drinking individuals (26, 41, 42). A recent study from Taiwan showed that the levels of inflammatory cytokines were elevated

during the early withdrawal phase (up to 4 days of abstinence), which was considerably ameliorated upon 4 weeks of abstinence (43). Regarding alcohol effects on HPA axis, the findings have been controversial, but varying results probably indicate different vulnerability factors and the extent of familiarization with alcohol (44). Corticosterone levels surges following acute alcohol intake by social drinkers in a dose-dependent manner, but this response is dampened in chronic AUD (45). In clinical AUD populations even without liver disease, levels of cytokines are typically increased compared to non-drinking individuals (26, 41, 42). As I will elaborate later, pro-inflammatory responses seen in chronic alcohol misuse are akin to those seen in MD.

Recent studies have shown long-term negative health outcomes in animals exposed to ethanol prenatally. In adult rats prenatally exposed to ethanol, the corticosterone reservoir was depleted and the cytokine production upon immune challenge was exaggerated (46). The ensuing low-grade inflammation correlated with memory deficits, which implicated a microglial role in fetal alcohol spectrum disorders (47). Also, substantial neuroinflammation caused by traumatic brain injury induced escalation of drinking in ethanol-habituated animals (48). Thus, CNS inflammation in both low and high grades changes alcohol drinking behavior. This phenomenon is clinically relevant.

Human experimental studies on the immune effects of ethanol consumption in healthy individuals are rare for obvious ethical reasons. A few endeavors have confirmed an early recruitment of immune cells following ethanol intoxication. Afshar et al. (49) gave a binge dose of alcohol to healthy men (0.9 g ethanol/kg body

weight) and women (0.8 g ethanol/kg body weight) and found a surge in the number of circulating monocytes, leukocytes, and natural killer cells—within 20 min of alcohol intake, which was followed by recovery toward baseline within 5 h. Thus, both innate and adaptive arms of the immune system are affected by alcohol. LPS induction of whole blood in the same sample showed fluctuations in inflammatory cytokines, and at 5 h, an anti-inflammatory state set in with elevated IL-10 and reduced IL-1 β levels. No sex differences in immune response were reported, although animal studies indicate that females are more vulnerable to the neuro-inflammatory effects of alcohol (50). For example, chronically ethanol-treated female mice expressed relatively greater levels of inflammatory mediators (iNOS and COX-2), cytokines (IL-1 β , TNF- α), gliosis processes, caspase-3 activation, and neuronal loss in the cerebral cortex compared to their male counterparts (50). This finding was confirmed in postmortem brain specimens of AUD individuals who had higher MCP-1 levels and increased microglial activation markers compared to controls (25). Human pharmacogenetic studies on alcohol dependence have coincidentally discovered several immune-gene polymorphisms as underlying excessive drinking (51).

NEUROIMMUNE ALTERATIONS IN DEPRESSION

The identification of immune disturbance in depressive illness (52) led to the “macrophage hypothesis of depression,” the proposition that inflammatory products of macrophage were responsible for depression (53). Since then, a consistent body of literature has confirmed that inflammatory processes are involved in the development and progression of depressive illness. Numerous studies have consistently documented positive associations of MD with C-reactive protein (CRP) and IL-6 (54). Meta-analyses have also supported depression’s associations with IL-1 β (55) and TNF- α (56, 57) as well as sIL-2 receptor (57). These associations held true for patient populations from the community as well as from clinical inpatient/outpatient settings. Patients with depression are found to have renormalized cytokine levels following treatment (58). Furthermore, several reports indicate longitudinal associations between CRP and subsequent development of depression (59), although an association was found in the opposite direction in a younger sample (60). Compelling evidence exists to suggest elevated levels of IL-6 as both a cause and a consequence of depression (61). In a 12-year prospective study of British civil servants, increased IL-6 levels at baseline predicted cognitive symptoms of depression at follow-up (62). These effects were reported to be consistent even after accounting for possible confounders such as, socio-demographics, behavioral and biological risk factors, health conditions, medication use, and baseline negative emotions. Recently, a population-based study from England ($N = 5,909$) showed positive associations between CRP and symptoms of fatigue, disturbed sleep, low energy, and low mood in a dose-response manner, a relationship that was absent in antidepressant medication users (63).

These novel findings quickly triggered drug trials using anti-inflammatory agents in depressive illness in humans. Notably, a proof-of-concept study examined infliximab, a TNF- α blocker in

patients with treatment-resistant depression. Twelve weeks after the initiation of therapy, infliximab reduced depressive symptoms by at least a half among patients with baseline hs-CRP > 5 mg/L, but not among those with lower baseline hs-CRP levels (64). Yet, another trial showed that adjunctive celecoxib, a selective COX2 inhibitor, was more effective in reducing depressive symptoms than sertraline alone in MD (65). Again, a reduction of serum IL-6 levels correlated very well with a reduction in depression score. However, the observation period was only 6 weeks. Several other drug trials using non-steroidal anti-inflammatory drugs have been conducted (66), mostly without rigorous patient selection. Significant methodological heterogeneity and publication bias make the reported positive efficacy less tenable.

One mechanism by which activated inflammatory cytokines (mainly IFN- γ and TNF- α) can aggravate depressive symptoms is through their induction of indoleamine 2,3-dioxygenase (IDO), an enzyme that metabolizes tryptophan along the neurotoxic kynurenine pathway (67, 68). IDO induction causes relative reduction in the availability of tryptophan, which is the amino acid precursor for serotonin synthesis. Tryptophan depletion and the neurotoxic metabolites produced downstream the kynurenine pathway may both trigger depression. In particular, peripheral macrophages and brain microglia preferentially metabolize kynurenine into anthranilic acid and quinolinic acid, both of which are NMDA receptor agonists and have potentially neurotoxic effects (69). Approximately, half of the cancer patients treated with IFN- α immunotherapy develops depression, and it was found that the severity of IFN- α -induced depression was related to the tryptophan degradation index (kynurenine to tryptophan ratio) along the kynurenine pathway (70). Studies also show a higher tryptophan degradation index ratio in individuals with MD compared to healthy controls (71, 72).

The failure of monoamine hypothesis to explain the delayed symptom relief in depression, despite early changes in brain monoamine neurotransmitter concentration following treatment, led to the emergence of the neurotrophic hypothesis of depression. It posits that chronic stress leads to reduced neurotrophic support to the brain limbic structures responsible for regulating mood and increases vulnerability to depression (73). Indeed, numerous studies have shown reduced serum levels of brain-derived neurotrophic factor (BDNF) in patients with MD compared to healthy controls (74–76), and evidence also exists to support renormalization of BDNF levels upon successful anti-depression interventions (74, 77). This process takes weeks to months. CNS and peripheral BDNF concentrations are altered in several mood and behavioral aberrations (74, 75, 78). Overexpressed pro-inflammatory cytokines in the brain and associated chronic neuroinflammation can lead to neurodegeneration and reduced neurogenesis, as indicated by decreased BDNF in multiple brain areas following LPS challenge (79). However, the cytokine network is rather complex, including pleiotropic effects that are sometimes paradoxical. For example, both neurotrophic and neurodestructive properties of IL-6 have been reported (80). Accordingly, circulating BDNF levels in depressed individuals were positively correlated with IL-6, but not with TNF- α (81). In recovering alcoholics, however, serum BDNF levels were positively correlated with IL-6 and TNF- α (82). Thus, the interaction

between inflammatory cytokines and BDNF remains an active area of research.

While the search for neuroimmune targets in depression continues, alternative medicine has also contributed to the field. Salidroside, a traditional Tibetan herbal product, known for its antioxidative and immunotonic effects, was administered to mice that were later exposed to LPS (83). The study revealed that salidroside could effectively ameliorate LPS-induced depression-like behavior while also attenuating the inflammatory cytokine and NF- κ B. Further investigations using polyphenolic compounds such as curcumin and resveratrol are underway to test the possible role of these agents in HPA axis modulation, hippocampal neurogenesis, and central monoamine homeostasis. Additionally, several other compounds related to immune regulation are of value: statins, polyunsaturated fatty acids, ketamine, TLR-inhibitors, glycogen synthase kinase-3 inhibitors, oleanolic acid analogs, and minocycline (84).

The most pressing caveat is that inflammation is neither necessary nor sufficient to cause depression, which means that activated inflammatory response would accompany only a subgroup of individuals with MD. Circulating and CNS levels of the inflammatory cytokines induced by alcohol are also modest, typically exceeding the levels in the healthy controls by a factor of 2–5 (24, 43). Unlike in purely inflammatory conditions, inflammatory markers in these low-inflammatory states rise only marginally, thus making interpersonal variations difficult to interpret (85). Nonetheless, a finding of sustained immune activation can connect depression as well as AUD with the often coexisting conditions of low-grade inflammation such as cardiovascular diseases, diabetes, fibromyalgia, multiple sclerosis, and cancer (86). A bulk of psychoneuroimmunological literature stems from correlational evidence, which is clearly inadequate to explain the depression pathophysiology and to subsequently proffer clinical interventions. Thus, it is high time that the theoretically embraced entity of “inflammatory cytokine-associated depression” (87) be phenotype based on relevant biological and clinical characteristics. Omics-based approaches highlighting systems biomedicine could be beneficial (88). Only such progress would lead to an enhanced understanding of comorbid conditions of MD.

NEUROIMMUNE DYSREGULATION IN AUD–DEPRESSION COMORBIDITY

It should be noted that immune perturbations presented in the previous sections that focused on inflammatory cytokines are only parts of several interacting biological systems that are ascribed to AUD and MD. The proposed interrelated inflammatory and neurodegenerative mechanisms responsible for the neurobiological changes in depression involve activated central and peripheral pro-inflammatory cytokine response, lowered levels of zinc and ω 3 polyunsaturated fatty acid overload, oxidative and nitrosative stress, tryptophan degradation along the kynurenine pathway, reduced neurogenesis, and increased neurodegeneration (89). A complex interaction between these processes produces neurobiology of depression and contributes to related brain disorders. For example, inflammatory cytokines in the brain are toxic to dopaminergic neurons and may precipitate

Parkinson’s disease (90). Two main factors contributing to the development of alcohol addiction are reinforcement (positive and negative) and neuroadaptation, both of which seem closely related to alterations in these processes, as has been elaborated in previous sections. Given the high rates of comorbidity and overlapping pathophysiological changes in various aspects of the neuroimmune system that accompany each disorder, it will be no surprise if neuroimmune changes in AUD–MD comorbidity are somehow coordinated (**Figure 2**).

Hypothalamic–pituitary–adrenal-axis hyperactivity and glucocorticoid receptor impairment are reliable findings in depression (91), and altered HPA axis regulation is a hallmark of hormonal dysbalance in AUD (92). The nature of HPA axis abnormality upon ethanol depends on various stages of the disease and ethanol dose. In chronic AUD cases, basal ACTH levels are elevated and stress- and cue-induced corticotropin and cortisol responses are suppressed (93). Alcohol withdrawal syndrome is characterized by symptoms of autonomic hyperactivity such as tremor, sweating, anxiety, agitation, nausea, and malaise. Symptoms also include disturbed sleep and depressed mood. Interestingly, blocking the hypothalamic corticotropin-releasing factor (CRF) ameliorates the dysphoric symptoms of alcohol withdrawal (94) and the increased stress responsiveness and associated anxiety-like behavior during protracted abstinence (95). CRF blockade in depression-like behavior in a mouse model was shown to reduce those symptoms through modulation of neuronal plasticity (96). Taken together, involvement of brain stress systems in neuroadaptive changes accompanying addiction and emotional circuitry provides a common interface for AUD- and MD-related neuronal changes.

Inflammatory cytokines are potent inducers of CRF and, therefore, negative affect during withdrawal and negative reinforcement during long-term abstinence could potentially arise from immune-mediated CRF activation. Glucocorticoids thus produced cause tryptophan degradation by activating hepatic degradation of tryptophan 2,3-dioxygenase (TDO), which, along with cytokine-induced IDO in the brain, once again produces metabolites biased toward the neurotoxic edge (97, 98). TDO enzyme is activated upon acute alcohol consumption, subsequently inhibited with chronic alcohol drinking, and again surges during ethanol withdrawal (99–101). The altered tryptophan metabolism reportedly lasts for several months into abstinence, as shown in a comparative study of 4 and 11 weeks of abstinence, wherein longer abstinence was related to increased kynurenine levels (102, 103). This could well be explained in terms of hyperactive stress response in concert with negative reinforcement, craving, and relapse. We reported increased tryptophan turnover with increased duration of abstinence (104). However, another study (105) showed that AUD individuals who abstained from alcohol for longer than two weeks, regardless of background variables, had much higher tryptophan levels compared to healthy controls. Literature also indicates a contradictory higher tryptophan and lower tryptophan degradation in depression, alongside activated pro-inflammatory pathway (104, 106, 107), but these findings are based on peripherally measured mediators and may not reflect brain levels. An overview of the few studies that have investigated neuroimmune mediators in the context of

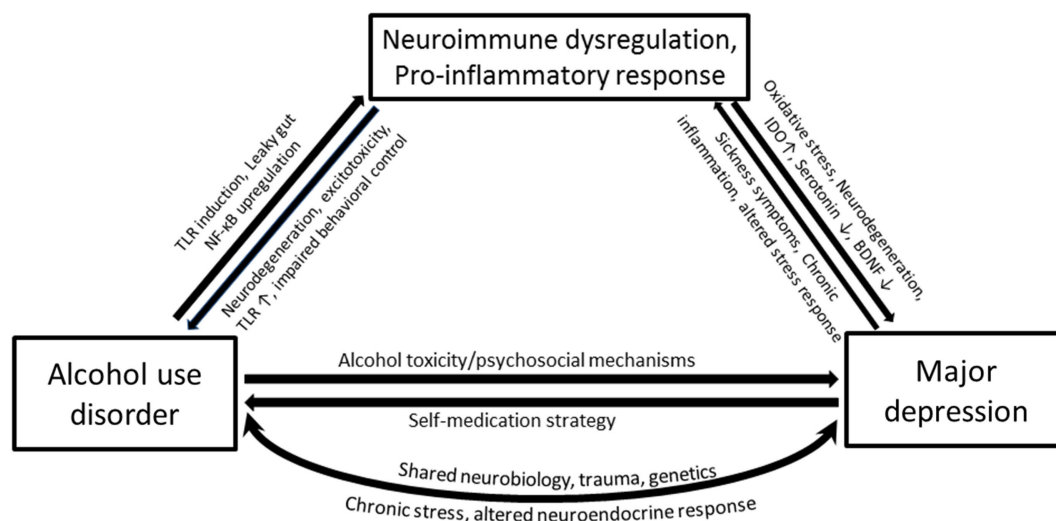


FIGURE 2 | Simplified pathways illustrating the potential mechanisms that underlie associations between alcohol use disorder (AUD) and major depression (MD), with a compelling neuroimmune contribution. Heavy alcohol drinking may render gut wall permeable to bacterial proteins such as lipopolysaccharide (LPS) through the activation of toll-like receptors. Ethanol and LPS upregulate the transcription factor NF- κ B and cause immune cells in the periphery as well as glial cells to produce pro-inflammatory cytokines. The pro-inflammatory cytokines within the brain activate the indolamine 2,3-dioxygenase enzyme, which metabolizes tryptophan away from serotonin production toward a potentially neurotoxic kynurenine pathway. MD accompanies altered monoamines, oxidative and nitrosative damage, and neurodegeneration. Depression is associated with chronic inflammatory conditions such as cancer and cardiovascular diseases, which together with sickness symptoms can feed the neuroimmune dysregulation causing further neurodegeneration, negative affect, and anxiety-like behavior and loss of behavioral control—all features characterized in AUD.

AUD–MD comorbidity are shown in **Table 1**. Further studies are needed to clarify these observations.

Neurotrophic changes in brain regions involved in depression and AUD are relevant considerations. As discussed in the previous section, depression is consistently associated with depleted BDNF. Indeed, neuroinflammation has an inhibitory effect on adult cortical and hippocampal neurogenesis, as evidenced by reduced BDNF expression concurrent to LPS-induced upregulation of inflammatory cytokines in rats (79). Similarly, chronic ethanol exposure in humans was accompanied by reduced BDNF expression in the hippocampus (108) as well as lower plasma BDNF protein levels (109). Reduced expression of BDNF in the hippocampus and cortical regions is a clear conjuncture for AUD and depression because these are critical target brain regions in both disorders. During the last decade, we (82) and others (110–113) have investigated BDNF in AUD patients. The findings of these studies indicate that neuronal repair initiates soon after the abstinence commences, and BDNF levels continue to rise over several months (109, 113–117). Rat models of alcoholism showed that augmenting BDNF actions by the use of the selective BDNF tyrosine kinase B receptor agonist (7,8-dihydroflavone) removed withdrawal-induced depression-like behavior (118). Depressive symptoms are observed during various stages of AUD. In many cases of AUD, associated depressive symptoms do not disappear even after sustained abstinence. Against this backdrop, bidirectional causality between AUD and MD has been demonstrated with a more robust association seen from AUD leading toward MD (10). These evidences suggest the existence of what might be considered *alcoholic depression* and that a biological explanation

for depression in AUD could be approached from the immune inflammatory and stress pathways. It remains to be shown how the neuroadaptive changes in recovering AUD individuals relate to depressive symptoms, and whether targeting key neuroimmune factors such as BDNF is a viable intervention option in AUD–MD comorbidity.

Immune signaling induces a range of physiological responses that are common to affective and behavioral disorders. Infection accompanies a TLR4-mediated pro-inflammatory response, indicated by raised IL-1 β , IL-6, and TNF- α levels, which leads to “sickness behavior” (119, 120). Sickness behavior is also observed upon psychological stress and exogenous cytokine administration such as during cancer treatment with IFN- α and includes physiological responses (e.g., fever and disturbed sleep) as well as behavioral symptoms (e.g., anorexia, reduced mobility, disappearance of body care activities and reduced social interaction) (119). Many of these features overlap with those of depression. Compelling evidence also suggests activated TLR4 signaling to accentuate alcohol drinking but also negative affect and anxiety-like behavior, especially during the withdrawal phase (121). Sickness symptoms wane away over several days; however, cytokine induction of these behavioral changes may persist as MD. Thus, a better understanding of the loop between immunity, the brain, and behavioral outcomes holds promises to newer approaches to intervene AUD–MD pathologies.

CONCLUSION AND FUTURE DIRECTIONS

The clinical realm of frequent comorbidity between AUD and MD requires an integrated psychobiological understanding that

TABLE 1 | Overview of studies investigating neuroimmune pathways in comorbid alcohol use disorder (AUD) and depression.

Reference, country	Subjects	Studied pathway/parameter	Main findings
Han et al. (110), South Korea	45 male inpatients with alcohol dependence	Growth factors	Depression score in AUD patients correlated positively with insulin-like growth factor, but not with nerve growth factor or BDNF
Plemenitaš et al. (122), Slovenia	101 alcohol abusing and 100 previously alcohol-dependent male inpatients abstinent for ≥2 years	Tryptophan metabolism; genetic association study	Genetic variability in tryptophan hydroxylase 2 (TPH2) gene associated with anxiety and, to some extent, with depression. TPH2 rs1843809 was associated with depressive and aggressive traits and TPH2 rs4290270 with depressive and anxiety traits
Neupane et al. (104), Nepal	153 male and 16 female AUD inpatients	Tryptophan metabolism	Concurrent depressive state related to counterintuitive higher tryptophan level and lower tryptophan degradation index. Tryptophan metabolism related to abstinence duration and AUD severity
Neupane et al. (82), Nepal	152 male AUD inpatients	BDNF	Concurrent depressive state in AUD related to lower BDNF serum levels. Among patients in controlled abstinence, history of binge drinking, and severe AUD associated with higher BDNF serum levels. Tumor necrosis factor- α (TNF- α) correlated with BDNF levels
Neupane et al. (107), Nepal	156 male and 20 female AUD inpatients	Cytokines	Higher serum levels of inflammatory cytokines [interleukin (IL)-6, TNF- α , IFN- γ], but not IL-10 among comorbid major depression (MD) group. Cytokine levels less increased in depression comorbid with greater severity of AUD than less severe AUD
Nedic et al. (111), Croatia	549 male and 126 female patients with alcohol dependence	BDNF; genetic association study	BDNF Val66Met polymorphism not related to depression in alcohol dependence
Su et al. (112), China	548 male Han Chinese with alcohol dependence	BDNF; genetic association study	The A allele of BDNF rs6265 was significantly overrepresented in alcohol-dependent patients with depression compared to patients with isolated alcohol dependence
Umene-Nakano et al. (113), Japan	13 male and 6 female inpatients with MD and alcohol dependence	BDNF	No significant difference was found in the serum BDNF levels of depressive patients with and without alcohol dependence. BDNF levels increased among responders to antidepressant medication (8 weeks), but not among non-responders

BDNF, brain-derived neurotrophic factor.

underlies these conditions. As presented in this review, mounting evidence supports neuroimmunological alterations, in particular activated immune responses (26), as a critical piece of the physiological link between AUD and MD. Neuroimmune gene induction in limbic brain regions increases negative affect, drug-seeking behavior, and loss of behavioral control (51). Diminished affect is a hallmark of depression, and anxiety-like behavior is pronounced in the withdrawal phase of alcohol addiction. Thus, mood symptoms as well as emotional and behavioral lability in AUD and MD appear to stem from neuroimmune mechanisms (21, 51, 87). The relative contribution of one phenomenon in the context of the other remains unclear. The current evidence is clearly inadequate to unravel the full scope of possible neuroimmune etiopathology of isolated AUD and MD, and an endeavor to attack their comorbidity may sound premature at this stage. However, this complexity should not be a hindrance to investigate these two disorders in their totality, because the relative neuroimmune contribution to each disorder may become clearer when one of the comorbid conditions vanishes. Prospective studies investigating longitudinal associations between changes in neuroimmune function and changes in depressive symptoms, drinking behavior, and treatment outcome are necessary. Furthermore, researchers in the field should be aware of the ethical obligation not to categorically exclude patients who have additional burden.

An extension of the research focus from isolated to comorbid disorders and from preclinical to clinical settings is conducive

to appraising the significant overlaps in manifestation of AUD and MD, as well as common biological perturbations in their development and maintenance. It is important to note that the neuroimmune approach alone would not be sufficient to elucidate the underlying complex etiopathology of AUD and MD, which strongly involves other genetic, epigenetic, and environmental factors. However, the neuroimmune approach would constitute an essential component of the systems biomedicine and be applicable to a significant proportion of patient populations. Equally important is the identification of intermediary processes that may determine the ultimate neuroimmune allostasis. Taken together, an exploration of a neuroimmune model for AUD–MD comorbidity provides a foundation for the development of more effective immune-based pharmacotherapy against these burdensome disorders.

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The author confirms being the sole contributor of this work and approved it for publication.

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Histological Architecture Underlying Brain–Immune Cell–Cell Interactions and the Cerebral Response to Systemic Inflammation

Atsuyoshi Shimada^{1*} and Sanae Hasegawa-Ishii²

¹ Department of Pathology and Laboratory Medicine, Central Hospital, Aichi Human Service Center, Kasugai, Aichi, Japan,

² Department of Pharmacology, Pennsylvania State University College of Medicine, Hershey, PA, USA

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*Correspondence:

Atsuyoshi Shimada
ats7@inst-hsc.jp

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Although the brain is now known to actively interact with the immune system under non-inflammatory conditions, the site of cell–cell interactions between brain parenchymal cells and immune cells has been an open question until recently. Studies by our and other groups have indicated that brain structures such as the leptomeninges, choroid plexus stroma and epithelium, attachments of choroid plexus, vascular endothelial cells, cells of the perivascular space, circumventricular organs, and astrocytic endfeet construct the histological architecture that provides a location for intercellular interactions between bone marrow-derived myeloid lineage cells and brain parenchymal cells under non-inflammatory conditions. This architecture also functions as the interface between the brain and the immune system, through which systemic inflammation-induced molecular events can be relayed to the brain parenchyma at early stages of systemic inflammation during which the blood–brain barrier is relatively preserved. Although brain microglia are well known to be activated by systemic inflammation, the mechanism by which systemic inflammatory challenge and microglial activation are connected has not been well documented. Perturbed brain–immune interaction underlies a wide variety of neurological and psychiatric disorders including ischemic brain injury, status epilepticus, repeated social defeat, and neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease. Proinflammatory status associated with cytokine imbalance is involved in autism spectrum disorders, schizophrenia, and depression. In this article, we propose a mechanism connecting systemic inflammation, brain–immune interface cells, and brain parenchymal cells and discuss the relevance of basic studies of the mechanism to neurological disorders with a special emphasis on sepsis-associated encephalopathy and preterm brain injury.

Keywords: choroid plexus, leptomeninges, bone marrow transplantation, endotoxemia, cytokine, sepsis-associated encephalopathy, preterm brain injury

INTRODUCTION

The central nervous system (CNS) used to be regarded as an immune-privileged organ (1). CNS immune privilege was experimentally defined by the fact that tissues that were rapidly rejected by the immune system when grafted in sites such as the skin, showed prolonged survival when grafted into the CNS (2). Isolation of the brain from the immune system by the blood–brain barrier (BBB)

and the lack of draining lymphatics also contributed to the idea of brain immune privilege (3).

However, recent data have dramatically altered such a viewpoint by demonstrating that the CNS interacts actively with the peripheral immune system and is therefore immune competent. Interactions between the CNS and the peripheral immune system are achieved *via* humoral factors, nerve fibers, and cell–cell interactions under both non-inflammatory and inflammatory conditions. In this article, we will focus on the tissue architecture and cellular components that enable cell–cell interactions between the brain and the immune systems in organisms. We discuss how these interactions are relevant to neurological diseases in which systemic inflammation underlies the pathogenesis.

NEW ASPECTS OF THE BRAIN–IMMUNE SYSTEM INTERACTION

The immune system plays an important role in maintaining the higher brain functions under non-inflammatory conditions. For example, severe combined immune deficiency mice and nude mice that are deficient in mature T cells exhibit cognitive deficits and behavioral abnormalities that are remedied by T cell restoration (4). Systemic depletion of CD4⁺ T cells leads to reduced hippocampal neurogenesis and impaired learning in the Morris water maze (5). Although there are no T cells present in the healthy brain parenchyma under non-inflammatory conditions, T cells accumulate in the meninges and express high levels of interleukin (IL)-4, which skews meningeal myeloid cells toward an anti-inflammatory phenotype during cognitive task performance (6, 7).

The so-called glymphatic system is a brain-wide perivascular pathway that consists of a paraarterial cerebrospinal fluid (CSF) influx route, a paravenous interstitial fluid clearance route, and an intracellular trans-astrocytic path that is mediated *via* aquaporin-4 (8, 9). The glymphatic system supports the exchange of CSF with interstitial fluid and plays a role in driving waste products by way of vectorial convective flow from the interstitium toward the paravenous space, where waste products may gain access to lymphatic vessels in the neck (8). Interestingly, natural sleep or anesthesia increases the volume of interstitial space by 60%, resulting in a remarkable increase in convective exchange of CSF with interstitial fluid, which propels the clearance of potentially neurotoxic waste products such as A β oligomers that accumulate during wakefulness (10).

More recently, functional lymphatic vessels that line the dural sinuses have been discovered by two groups independently (11, 12). These sinus-associated lymphatic vessels are able to carry both fluid and immune cells from the CSF (11). The dural lymphatic vessels absorb CSF from the adjacent subarachnoid space and brain interstitial fluid *via* the glymphatic system (12). Dural lymphatic vessels transport fluid into deep cervical lymph nodes *via* foramina at the base of the skull. Therefore, the dural lymphatic vessels and glymphatic system may act together to drive A β from the brain (13). In addition, the dural lymphatic system presumably provides architecture that supports immune cell dynamics during the performance of higher functions of the brain under

non-inflammatory conditions. In this regard, impaired functions of the glymphatic system or dural lymphatic system may cause neurodegenerative dementia such as Alzheimer's disease (AD).

SITES OF INTERCELLULAR INTERACTION BETWEEN BRAIN CELLS AND IMMUNE CELLS

Bone Marrow Transplantation (BMT) to Search for the Brain–Immune Interface

Although the brain is now known to actively interact with the immune system under non-inflammatory conditions, the site of cell–cell interaction between brain parenchymal cells and immune cells remains an open question. It was reported that following BMT by intravenous injection of donor cells, donor-derived cells were distributed throughout the brain parenchyma, and differentiated into ramified microglia (14, 15). However, it is now recognized that postnatal hematopoietic progenitors do not significantly contribute to microglia renewal or homeostasis in the adult brain (16). Other investigators have claimed that the donors' bone marrow-derived cells are found chiefly in association with blood vessels and are only rarely found in the brain parenchyma (17–19).

A novel BMT procedure has since been developed that is called “intra-bone marrow (IBM)-BMT” (20). In this procedure, BMCs are collected from the marrow of the donors' long bones by perfusion, and the entire BMCs are injected directly into the bone marrow cavity of the recipients instead of being injected intravenously (20, 21). The advantage of this IBM procedure is that it facilitates the transplantation of mesenchymal stem cells (MSCs) as well as of hematopoietic stem cells (HSCs) from the donor into the recipient (22). Two to six days after BMT, higher numbers of both HSCs and MSCs were reported in the bone marrow cavity of chimeras prepared by using the IBM procedure than those resulting from a conventional intravenous procedure (23–28).

Leptomeninges, Choroid Plexus Stroma, Perivascular Space, and Circumventricular Organs (CVOs) As Brain–Immune Interfaces

In our first set of experiments described in the present article that has been originally published in Ref. (29), we used bone marrow-chimeric mice prepared by the IBM procedure to investigate the distribution and time-dependent changes in the density of bone marrow-derived cells as well as in their differentiation in the brain under non-inflammatory conditions (29). Bone marrow-derived cells start to appear in the leptomeninges and choroid plexus stroma at 2 weeks after BMT, and to appear in the brain perivascular spaces at 4 weeks after BMT in the bone marrow chimera. In the leptomeninges, bone marrow-derived cells exhibit round or spindle-shaped morphology. Spindle-shaped cells are located along the pia and in close apposition to subpial astrocytic endfeet. Bone marrow-derived cells that have entered the perivascular space, which is a narrow space between the outer

surface of capillary walls and the brain parenchymal surface, are situated in close apposition to perivascular astrocytic endfeet. In the choroid plexus stroma, bone marrow-derived cells with a round to ovoid morphology often appear in clusters. Isolated, scattered bone marrow-derived cells exhibit a spindle-shaped morphology without ramified processes. Importantly, leptomeninges, perivascular spaces, and choroid plexus stroma share tissue components that are contiguous due to the fact that these brain regions are derived from the same developmental origin. Therefore, these spaces are one of the major interfaces between the immune system and the brain.

At 1 month after BMT, bone marrow-derived cells enter structures that lack a BBB and that are collectively named CVOs, including structures such as the median eminence, posterior pituitary, subfornical organ, pineal gland, and area postrema. Most bone marrow-derived cells in the CVOs are closely associated with blood vessels and exhibit elongated cell bodies with rare ramified processes. Therefore, CVOs are also interfaces between the immune system and the brain.

The Attachments of Choroid Plexus As a Novel Brain–Immune Interface

A group of brain regions that has not been a focus of attention from scientists in the field is a group of particular brain parenchymal regions that is populated by bone marrow-derived cells (29). These regions are relatively small in size and discrete. Bone marrow-derived cells in these particular regions frequently exhibit multiple ramified processes (ramified marrow-derived cells). All ramified marrow-derived cells express Iba-1, a marker for myeloid cells (monocyte/macrophage lineage) (29). None of the ramified marrow-derived cells express markers for astrocytes, oligodendrocytes, or neurons. In chimeric mice in which the IBM procedure is used, most of these ramified marrow-derived cells start to appear at 4 months after BMT. The density of ramified marrow-derived cells increases thereafter, as a function of post-BMT time, for up to 8 months, which is the longest time point that we have examined so far (29). Ramified marrow-derived cells are distributed with the highest density (>16 cells/mm²) in the habenula and the brain stem cochlear nucleus, followed by the medial amygdala and the bed nucleus of stria terminalis (8–16 cells/mm²). They are distributed with moderate densities (4–8 cells/mm²) in the piriform cortex, thalamus, and cerebellar cortex. In the ventral hippocampus, superior colliculus, hypothalamus, and midbrain tegmentum, ramified marrow-derived cells are distributed at relatively low densities (2–4 cells/mm²). The regions populated by ramified marrow-derived cells contain junctions at which the choroid plexus is attached to the brain parenchyma (29). In the lateral ventricle, the choroid plexus is formed by papillary protrusion of the leptomeninges toward the inside of the ventricular cavity, where they bridge the stria terminalis and medial amygdala. The choroid plexus stroma shares tissue components with the leptomeninges and consists of loose connective tissue containing many capillaries. The choroid plexus epithelial cells are a continuation of the ependymal cells. At the edge of the stria terminalis or medial amygdala, the brain parenchyma is extremely thin and consists of loose wavy fibrous processes of astrocytes

that are located in the narrow channel between the ependyma and the pia (**Figure 1**). We call this particular structure that consists of ependyma, loose glial tissue, and pia that connects the brain parenchyma and the choroid plexus the “attachments of choroid plexus.”

Bone marrow-derived cells exhibit a round to ovoid morphology in the choroid plexus stroma, whereas bone marrow-derived cells are spindle-shaped and often exhibit a tortuous appearance when they are inside the attachments of choroid plexus. Once these cells enter the brain parenchyma, they cluster in a relatively small area and differentiated into ramified morphology (**Figure 2**) (30). These observations raise the possibility that bone marrow-derived cells enter the brain parenchyma through the attachments of choroid plexus. The sites of intercellular interaction between brain cells and immune cells that are based on the results of our IBM-BMT studies are summarized in a cartoon illustration in **Figure 3**.

There are presumably some molecular cues that enhance the migration of bone marrow-derived cells from the choroid plexus to the brain parenchyma. One such candidate cues is the CX3CL1–CX3CR1 signaling system. CX3CL1 (or fractalkine) is strongly expressed in the attachments of choroid plexus in both BM-transplanted and non-treated mice in a similar manner (30). CX3CL1 is constitutively expressed in a portion of the elongated cytoplasmic processes that emanate from astrocytes whose cell bodies are located in the adjacent brain parenchyma. Monocyte-derived cells in the choroid plexus and microglia in the brain parenchyma are known to express CX3CR1 (32), which is a highly specific receptor for CX3CL1 (33). CX3CL1 is a transmembrane molecule with a unique CX3C motif whose expression is induced by inflammatory cytokines (34). In addition to its functions as a cell adhesion molecule (33), membrane-anchored CX3CL1 can be released from the cell surface by cleavage and shedding mediated by A Disintegrin And Metalloproteinase (ADAM)10 and ADAM17 (35, 36). Once released, soluble CX3CL1 functions as a chemoattractant for cells bearing CX3CR1 (33, 34). The CX3CL1–CX3CR1 signaling system is also known to be required for physiological trafficking of circulating monocytes to peripheral organs such as lung (37) and small intestine (38). Therefore, bone marrow-derived cells that are in the process of lining up along the narrow channel of the attachments of choroid plexus may make cell–cell contact with astrocytic processes that express CX3CL1, resulting in their trafficking into brain parenchyma.

ENDOTOXEMIA-INDUCED CYTOKINE-MEDIATED RESPONSES OF HIPPOCAMPAL ASTROCYTES TRANSMITTED BY CELLS OF THE BRAIN–IMMUNE INTERFACE

Endotoxemia-Induced Impairments in Brain Higher Functions

The immune system modulates the functional and behavioral processes of the CNS under inflammatory conditions (39). Exposure to pathogens such as lipopolysaccharide (LPS) stimulates the peripheral immune system and induces inflammatory

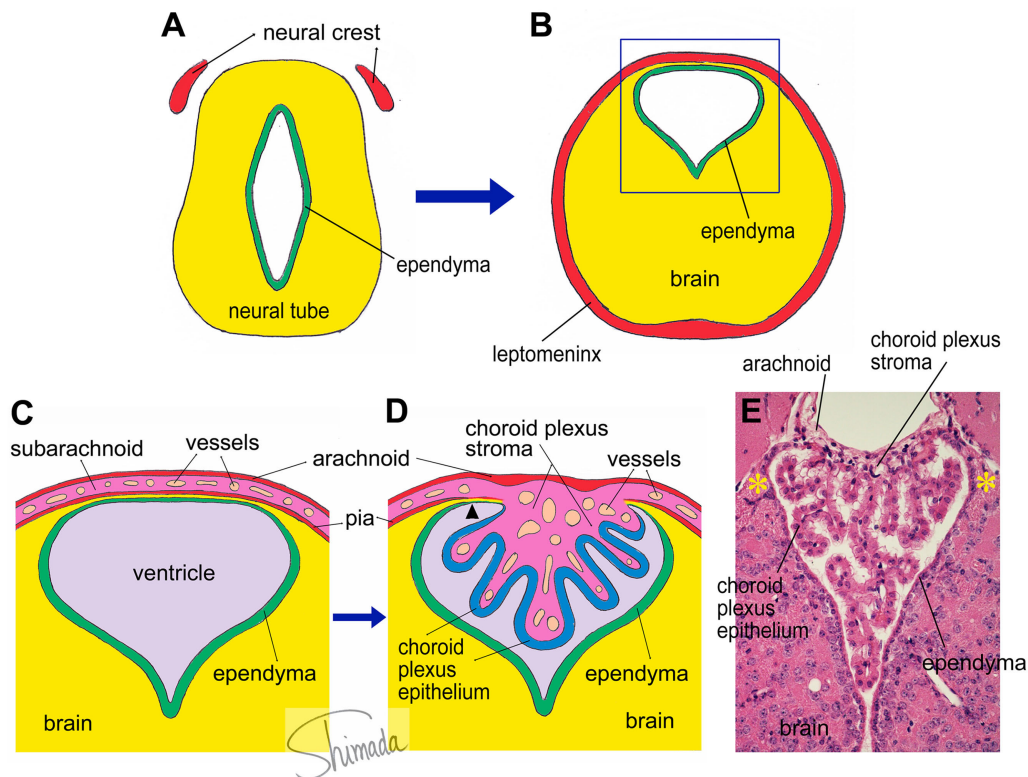


FIGURE 1 | Schematic illustration of choroid plexus morphogenesis. (A,B) The neural tube contains the future cerebral ventricle and that the leptomeninx is derived from the neural crest. In particular parts of the neural tube, the cerebral ventricle is shifted to the dorsal end of the brain parenchyma, from which the choroid plexus is formed. **(C)** An enlarged view of the square inset indicated in panel **(B)**. The choroid plexus is formed as a papillary protrusion of the subarachnoid stromal tissue **(D)**. Therefore, the choroid plexus stroma shares tissue components with the subarachnoid space and consists of loose connective tissue containing many capillary blood vessels. The choroid plexus epithelium is a continuation of the ependyma. At the edge of the choroid plexus, the brain parenchyma is extremely thin and located in a narrow channel between the ependyma and pia (arrow head). We call this particular structure the “attachments of choroid plexus.” The real histological architecture that is represented by the schema in panel **(D)** is indicated by a photoimage of a paraffin-embedded section of the mouse choroid plexus from the third ventricle stained with hematoxylin and eosin **(E)**. Note the presence of the “attachments of choroid plexus” in both sides (*).

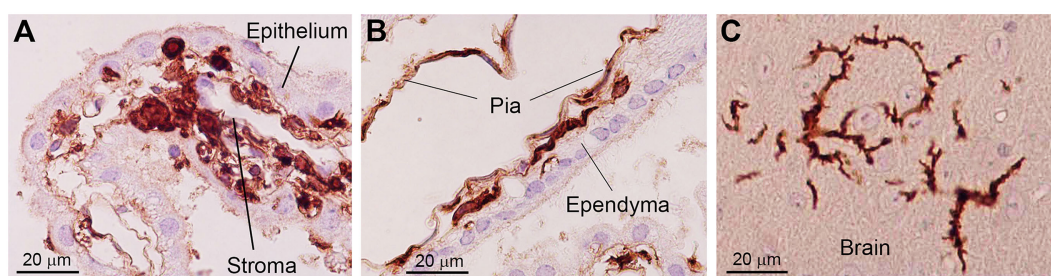


FIGURE 2 | Photoimages indicating changes in the morphology of bone marrow-derived cells in the brain. In the choroid plexus stroma, bone marrow-derived cells exhibit a round to ovoid morphology **(A)**. When bone marrow-derived cells are located inside the attachments of choroid plexus, they are spindle-shaped and often exhibit a tortuous appearance with sharp bends **(B)**. Once these cells enter the brain parenchyma, they differentiate into a ramified morphology **(C)**. Immunohistochemical staining with an anti-GFP antibody. Nuclei are counterstained with hematoxylin. This figure is a modification of copyrighted material permitted by Academic Press (29), License Id: 3917441104164.

responses with the elevation of proinflammatory cytokines such as IL-1 (40, 41), IL-6, and tumor necrosis factor (TNF)- α (42). Increased levels of proinflammatory cytokines in peripheral tissues lead to the local synthesis of proinflammatory cytokines within the brain parenchyma (43–45), resulting in behavioral

alterations such as sickness behavior (46–48), impaired learning (49), and depressive-like behavior in mice (50).

The hippocampus is one of the targets for behavioral changes induced by systemic inflammatory challenge. Types of memory tasks sensitive to peripheral LPS administration including a

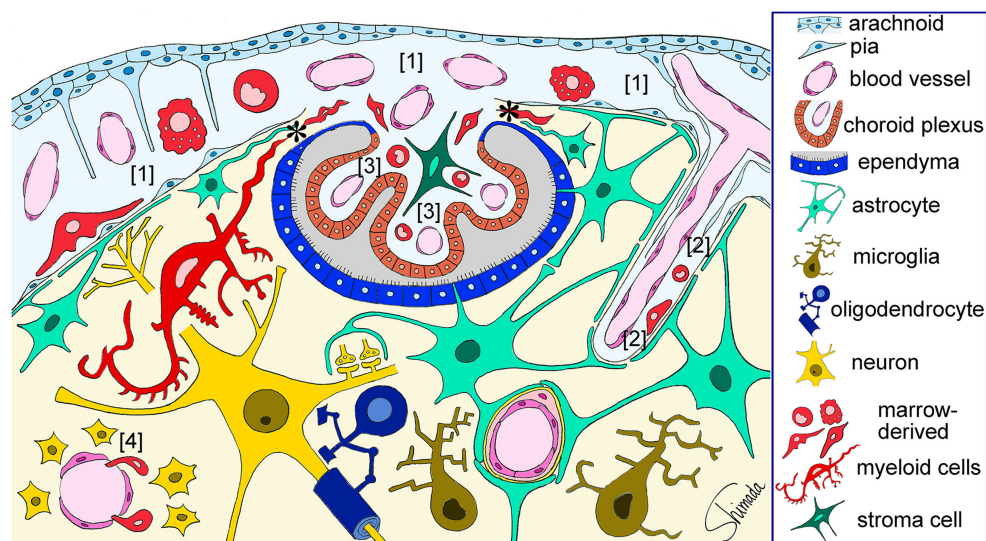


FIGURE 3 | Schematic illustration of the sites of brain–immune cell–cell interaction. Red-colored cells represent bone marrow-derived myeloid cells that are located in the subarachnoid space [1], perivascular space [2], and choroid plexus stroma [3], all of which are contiguous. Bone marrow-derived cells enter the subarachnoid space through fenestrated blood vessels and can be localized in close apposition to the pia and subpial astrocytic endfeet that are a part of the brain parenchyma. Marrow-derived cells in the perivascular space can be localized in close apposition to perivascular astrocytic endfeet. Marrow-derived cells can cross the loose blood vessel walls of the circumventricular organs [4] and enter the parenchyma. Marrow-derived cells in the choroid plexus stroma can migrate into the brain parenchyma through the attachments of choroid plexus (*) without being blocked by the blood–brain barrier. These cells dramatically change their shape into a ramified morphology once they enter the parenchyma. Therefore, immune cells can interact with brain parenchymal cells via at least four types of histological architecture. This figure is a modification of copyrighted material permitted by Nature Publishing Group (31), License Id: 3917450680042.

spatial water maze task (51), contextual fear conditioning (52), context discrimination memory (53), etc. are dependent on hippocampal function. However, LPS does not cross the BBB unless it is pathologically impaired (54). The amount of LPS entering brain parenchyma is only about 0.025% of an intravenously administered dose, which suggests that most effects induced by acute peripheral administration of LPS are not mediated through receptors expressed by brain parenchymal cells (55). Thus, how LPS outside the BBB mediates changes inside the brain is one of the most important remaining contemporary questions. Four possible routes by which the CNS and immune system might interact with each other have been proposed (56): a neural route *via* sympathetic or vagus nerves (57), CVOs, transport by cellular components that form the BBB (58), and secretion by vascular endothelial cells (58).

Microglial Involvement in the Communication of the Immune System with the Brain

Microglia are known to be activated by systemic LPS administration (59). Intravenous injection of LPS (1–5 mg/kg) into rats induces a morphological transition of microglia to macrophage-like cells in the hypothalamus, thalamus, and brainstem 8–24 h after LPS injection (60). A systematic review of 51 studies that performed animal experiments has reported a relationship between systemic inflammation and microglial activation (61). Most studies have shown that microglia are activated time-dependently following single systemic challenge of mice and

rats with LPS. What is required to induce CNS inflammatory responses during endotoxemia is the expression of toll-like receptor 4 (TLR4) on non-hematopoietic, intracranial resident cells located in the leptomeninges, choroid plexus and CVOs, cells along blood vessels, ependymal cells, and parenchymal microglia (62). Interestingly, non-parenchymal cells that are involved in sustained brain inflammation are located at the brain–immune interface as defined by our own studies using BMT (29, 30).

Depending on the experimental paradigm, activated microglia induced by systemic LPS administration can be neuroprotective. Daily intraperitoneal (i.p.) injections of LPS (1 mg/kg) for four consecutive days induce microglial activation that is detectable 24 h after the last injection, whereas microglial activation is not detectable at 24 h after a single i.p. injection of LPS (1 mg/kg) (63). Microglia thus activated by four daily i.p. injections of LPS are skewed toward an M2-like phenotype and participate in neuroprotection against experimental brain injury (63). It has been demonstrated that the activation of microglia induced by this LPS pretreatment paradigm is also independent of TLR4 on hematopoietic cells (63). Nevertheless, the mechanism connecting systemic inflammatory challenge and microglial activation has not been well documented (61).

Importance of Astrocytic Endfeet in the Endotoxemia-Induced Hippocampal Responses

In our second set of experiments described in the present article that has been originally published in Ref. (31), we induced

endotoxemia by a single i.p. injection of LPS at a dose of 3 mg/kg into mice, and by using Luminex multiplex assay technology, we identified cytokines that showed a change in concentration in the hippocampus and spleen. We then immunohistologically identified cells involved in the elevation of hippocampal cytokine levels (31). The concentration of at least 10 cytokines increases in the hippocampus in response to endotoxemia. Leptomeningeal stromal cells, choroid plexus stromal cells, and choroid plexus epithelial cells produce CC-motif ligand (CCL2), CXC-motif ligand (CXCL1), CXCL2, and IL-6 transiently at 4 h after LPS injection. Hippocampal vascular endothelial cells produce CXCL1 at 4 h after LPS injection. Importantly, these cells are located at the brain–immune interface but not in the brain parenchyma.

Cytokine whose concentrations are increased in the hippocampus at later than 4 h and at up to 24 h after LPS injection are produced chiefly by brain parenchymal astrocytes (31). Based on the fact that astrocytic endfeet express receptors for CCL2, CXCL1, CXCL2, and IL-6, it is considered that astrocytes are exposed to these cytokines *via* endfeet that are located in close apposition to cytokine-producing cells at the brain–immune interface. Stimulation of astrocytic endfeet with cytokines released by interface cells is very likely to result in inducing astrocytes to release another group of cytokines including CXCL10, CCL11, and granulocyte-colony stimulating factor (G-CSF) into the hippocampal interstitium (31). An increase in cytokines in the hippocampus occurs later than in the spleen, suggesting that the brain requires more steps than the spleen to produce cytokines in response to endotoxemia.

Astrocytes can produce CCL11 in response to endotoxemia. CCL11 in the brain is known to impair learning and memory (64), although this chemokine is known as a strong eosinophil chemoattractant that mediates allergic diseases. CCL11 also promotes the migration of microglia and induces microglia to produce reactive oxygen species, thereby enhancing excitotoxic neuronal death (65). Thus, astrocyte-derived CCL11 in our model may exert harmful effects on the hippocampal functions, leading to behavioral changes and memory impairment.

Astrocytes can also produce G-CSF in response to endotoxemia. Although G-CSF exerts neuroprotective actions on cerebral ischemic damage by promoting neuronal progenitor responses (66, 67), systemic injection of G-CSF into uninjured mice enhances the proliferation of microglia in the intact hippocampus (68). The latter observation suggests that G-CSF may act as a growth factor for microglia. Thus, astrocyte-derived G-CSF in our model may enhance the proliferation of microglia in the hippocampus, resulting in behavioral changes and memory impairment.

CXCL1 is produced by vascular endothelial cells. CXCR2, a receptor for CXCL1, is expressed on astrocytic perivascular endfeet. Given that cell–cell interactions with cytokines as mediators occur between cells located close to each other and that vascular endothelial cells are in close apposition to perivascular astrocytic endfeet that cover most of the perivascular surface of the cerebral microcirculation, a relationship between CXCL1 production by endothelial cells and CXCR2 expression by astrocytic endfeet appears reasonable. In the same manner, CCL2, CXCL1, CXCL2, and IL-6 are produced by leptomeningeal stromal cells, and the

receptors for these cytokines are expressed on subpial astrocytic endfeet. A relationship between these cytokines and their receptors also appears to be reasonable given the close apposition between leptomeningeal cells and subpial astrocytic endfeet that line the entire brain surface.

In addition to these cell–cell interactions, choroid plexus epithelial cells may also release cytokines into the ventricular space and choroid plexus stromal cells may release cytokines into the subarachnoid space. A report in which the flow of subarachnoid CSF into and through the brain interstitium was investigated (8) has indicated that fluorescent tracers with low molecular weight (759 Da) infused in subarachnoid CSF move quickly throughout the brain interstitium. Tracers with intermediate molecular weight (3 kDa) infused in subarachnoid CSF concentrate in the paravascular space but also enter the brain interstitium to a limited extent from the paravascular space and from the pial surface. By contrast, large molecular weight tracers (2,000 kDa) infused in subarachnoid CSF are confined along paravascular space and do not enter surrounding interstitial space. Since the molecular weights of CCL2, CXCL1, CXCL2, and CXCL10 are approximately 7–8 kDa and that of IL-6 is approximately 18 kDa, expected movements of these cytokines in subarachnoid space are likely similar to those of tracers with intermediate molecular weight. Therefore, these cytokines may infiltrate the interstitium of paravascular and subpial regions.

Another possible pathway for cytokine flow from the choroid plexus into the hippocampus involves the attachments of choroid plexus (29, 30). Given that the hippocampus holds the attachments of choroid plexus at the fimbria, cytokines released by choroid plexus stromal cells may flow into the fimbria through the attachments of choroid plexus.

Thus, cells of the brain–immune interface respond to endotoxemia with cytokine-mediated signals earlier than hippocampal parenchymal cells. In the parenchyma, astrocytes play a key role in responding to these signals by using endfeet with cytokine receptors that are located in close apposition to the interface cells. These results are summarized in a cartoon illustration in **Figure 4**. How the responses by astrocytes are relayed to microglia awaits further studies.

CLINICAL RELEVANCE OF BASIC STUDIES OF THE STRUCTURE AND FUNCTION OF THE BRAIN–IMMUNE INTERFACE

Perturbed Brain–Immune Interaction in a Wide Variety of Neurological and Psychiatric Diseases

Bone marrow-derived cells that enter the brain parenchyma increase in number in animal models of ischemic brain injury (69–71) and status epilepticus (72, 73), as well as in animal models of neurodegenerative diseases such as AD (74), Parkinson's disease (PD) (75, 76), and amyotrophic lateral sclerosis (ALS) (77, 78). In experimental allergic encephalomyelitis, the choroid plexus is known as the CNS lymphocyte entry point (79). In a

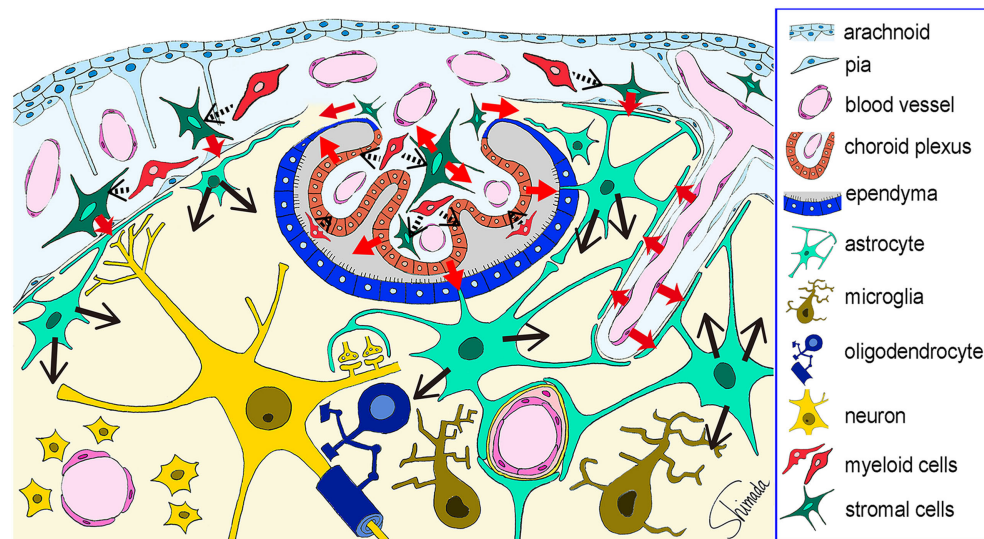


FIGURE 4 | Schematic illustration of the possible cytokine flow pathway by which systemic inflammation changes the brain cytokine microenvironment. In response to systemic inflammation induced by intraperitoneal injection of lipopolysaccharide (LPS), hippocampal vascular endothelial cells, leptomeningeal stromal cells, choroid plexus stromal cells, and choroid plexus epithelial cells produce early cytokines (such as CCL2, CXCL1, CXCL2, and IL-6) at 4 h after LPS injection (red arrows). The receptors for these cytokines are expressed by astrocytic endfeet that are localized in close apposition to vascular endothelial cells and leptomeningeal cells. Thus, cytokine-mediated cell–cell interactions occur between the endothelial cells and the perivascular astrocytic endfeet, and between the leptomeningeal stromal cells and the subpial astrocytic endfeet. In addition, cytokines produced by choroid plexus stromal cells and epithelial cells may flow into the cerebrospinal fluid, and those produced by choroid plexus stromal cells may also flow into the brain parenchyma via the attachments of choroid plexus (red arrows). Thereafter, astrocytes produce late cytokines (such as CCL11, CXCL10, and granulocyte-colony stimulating factor) at 4–24 h after LPS injection (black arrows). An initial step in the activation of stromal cells in the leptomeninges and choroid plexus, and in the activation of choroid plexus epithelial cells, may be triggered by nearby myeloid cells, although this remains to be proven (black arrows with dotted line). This figure is a reproduction of copyrighted material permitted by Nature Publishing Group (31), License Id: 3917450680042.

stroke model, bone marrow-derived perivascular, meningeal, and choroid plexus macrophages play an integral role in the inflammatory cascade (80). Repeated social defeat (81) and LPS-induced peripheral immune activation increases recruitment of bone marrow-derived monocytes into the brain (82). Recruitment of bone marrow-derived cells with the monocyte/macrophage lineage into the brain parenchyma is also enhanced in the senescence-accelerated mouse prone 10 (SAMP10), an animal model of human brain aging (83). The diencephalic structures located along the midline at the dorsal and ventral edges such as the choroid plexus epithelium, ependyma, astrocytic processes in the attachments of choroid plexus, periventricular astrocytes, tanycytes, and neurons of the median eminence and hypothalamus contribute to elevated tissue concentrations of CXCL1, CCL11, G-CSF, and CXCL10 in the brain. Changes in the cytokine profile accelerate the dynamics of immune cells migrating from the bone marrow to the diencephalon in SAMP10 mice.

Microglia are thought to be responsible for neuronal cell death in AD, PD, and ALS (84). Most published articles on microglia in these neurodegenerative diseases link activation of microglia with proinflammatory cytokine production (85). However, the presence of “dystrophic (senescent) microglia” in aged human brain has been proposed more recently (86). Given that microglia are neuroprotective, it is reasonable to consider that aging-related, progressive microglial degeneration, and loss of microglial neuroprotection rather than induction of microglial

activation contributes to the onset of sporadic AD (86). This idea is also supported by our own findings on microglial aging in SAMP10 mice (87).

Proinflammatory status associated with cytokine imbalance is involved in a wide variety of psychiatric diseases such as autism spectrum disorders (ASDs) (88), schizophrenia (89), and depression (90). Autopsy findings of postmortem brains from individuals with ASD have indicated prominent microglia activation as well as increased production of inflammatory cytokines such as interferon (IFN)- γ , IL-1 β , IL-6, TNF- α , and CCL2 in the brain tissue and CSF (88). Schizophrenia is associated with disruption of the cytokine milieu and the propensity for the production of proinflammatory cytokines (89). Acute psychotic episode is associated with increased serum concentrations of IL-6, TNF- α , IL-1 β , and IFN- γ (89). Patients with major depression exhibit increased serum/plasma concentrations of IL-6 and C-reactive protein as well as elevated levels of IL-1 β and TNF- α in peripheral blood and CSF (90).

Unlike multiple sclerosis, the archetypal inflammatory disease of the CNS in which an immune assault on the brain and spinal cord damages myelin sheaths and axons, most neurodegenerative and psychiatric diseases lack remarkable inflammatory cell infiltrates in the CNS (84, 91). Although monocytes and some other immune cells such as CD4+ and/or CD8+ T cells infiltrate the brain in neurodegenerative diseases, all of these immune cells appear in small numbers (84). Therefore, the

involvement of immune abnormalities in aging and neurodegenerative diseases as well as psychiatric disorders is associated with imbalance in cytokine milieu rather than brain infiltration of inflammatory cells.

Sepsis-Associated Encephalopathy (SAE)

Sepsis is the excessive systemic inflammatory reaction of an organism in response to an infection (92, 93). Septic encephalopathy or SAE is a multifactorial syndrome, which is characterized as diffuse brain dysfunction such as delirium, cognitive impairments, and loss of consciousness (94, 95). Importantly, patients develop SAE without clinical or laboratory evidence of direct brain infection or other types of encephalopathy (e.g., hepatic or renal encephalopathy) (94, 95). Instead, SAE is induced in the brain of an organism as a result of responses to systemic inflammatory changes. SAE is the most frequent type of encephalopathy encountered in the intensive care unit (92) and is present in about 20–30% of patients with sepsis. SAE often occurs before the failure of other organs (96) and, when present, it increases the mortality rate (97). In spite of the clinical importance of SAE, there presently exists no effective therapy that has been developed to specifically treat SAE in clinical settings (92), and only supportive therapy for the underlying disease is administered.

Although a case–control study in which the brain tissue of 13 patients with sepsis was compared with that of 17 controls reported a significant increase in the number of CD68-immunopositive activated microglia in the gray matter of septic patients (98), pathophysiological mechanisms of SAE have been proposed based chiefly on data obtained from experimental models. Disruption of the BBB due to loosened tight junctions of endothelial cells and detachment of the pericytes of hippocampal capillaries may play a role in SAE (99). Upregulation of intercellular adhesion molecule 1 during SAE (100) together with increased BBB permeability may induce circulating monocytes to adhere to the endothelium and pass into the surrounding tissues, where they proliferate and expand the population of perivascular macrophages (96).

In relatively early phase of sepsis, possible changes in the brain microcirculation may cause hypoxia–ischemia, which may lead to SAE through the upregulation of inflammatory gene transcripts including transcripts of TNF- α , IL-1 β , and inducible nitric oxide synthase. TNF- α has been suggested to be a key inflammatory mediator. TNF- α can be produced intrinsically in the brain, it regulates aquaporin, and it alters the transport of water into the brain resulting in edema (101). Cytokines, together with reactive oxygen species and nitric oxide, can also mediate a decrease in mitochondrial ATP generation, resulting in an energy deficit in early sepsis (102–105). Another possibility is that there is an elevation in the levels of complement activation products during sepsis, which can cause brain tissue injury, and which, again, occurs *via* the release of cytokines (106).

Our experimental data indicating that cells of the brain–immune interface respond to endotoxemia with cytokine-mediated signals earlier than brain parenchymal cells and that parenchymal astrocytes play a key role in responding to these signals by using cytokine receptors on endfeet located in close apposition to the interface cells (31) would contribute to an understanding of the initial events leading to SAE.

Preterm Brain Injury

Neonatal mortality and morbidity has been improved in the past half century. However, brain injury that occurs during the perinatal period is still a common cause of lifelong neurodevelopmental disabilities including cerebral palsy. The rates of cerebral palsy are not declining and may even be increasing in some western countries (107, 108). Therefore, the pathophysiology of perinatal brain injury remains a point of scientific debate. Although the etiology may be multifocal, hypoxia–ischemia, infection/inflammation, and excitotoxicity are considered important causes of perinatal brain injury (109). Experimental studies have indicated that antecedents such as infection/inflammation, intrauterine growth restriction, or preexposure to hypoxia modulate brain vulnerability (110, 111). Very recently, one of the mechanisms underlying the causal relationship between maternal immune activation and autistic behavioral abnormalities in the offspring has been revealed (112). Maternal immune activation increases IL-6 in the serum of pregnant mice, which leads to an increase in T_H17 cells in maternal blood. These cells release IL-17, which crosses the placenta and increase the expression of IL-17 receptors in the offspring's brain. This phenomenon in turn leads to autistic abnormalities in the offspring.

Brain injury evolves over time. Acute cerebral hypoxia/ischemia serves to cause depletion of tissue energy reserves, resulting in primary insult, which is often followed by transient restoration of energy metabolism upon reoxygenation. Thereafter, delayed or secondary cerebral energy failure parallels a decrease in tissue glucose metabolism and development of cell injury (113). Even after secondary cell death has subsided, effects on the brain persist including sensitization to inflammation or injury, increased seizure susceptibility, impaired oligodendrocyte maturation and myelination, and persistent inflammation and gliosis (114–116). Tertiary brain damage is defined in such situations as “injury caused by a long-persisting process following brain insult that worsens outcome, predisposes to further injury, or prevents repair/regeneration” (109). Tertiary brain injury underlies cerebral palsy (116), schizophrenia (117), and ASD (118).

An estimated 500,000 babies are born preterm (before 37 completed weeks of gestation) every year in the United States. Chorioamnionitis is a common cause of preterm birth. Clinical chorioamnionitis is characterized by maternal fever, leukocytosis, tachycardia, uterine tenderness, and prerupture of membranes (119), whereas subclinical chorioamnionitis is asymptomatic and is defined by inflammation of the chorion, amnion, and placenta (120). Subclinical chorioamnionitis is more common than clinical chorioamnionitis. Chorioamnionitis is often associated with a fetal inflammatory response syndrome that is defined by increased systemic inflammatory cytokine concentrations, funisitis, and fetal vasculitis (120). The fetal inflammatory response syndrome leads to a long-term adverse outcome such as impaired fetal cardiac function, chronic lung disease, retinopathy of prematurity, and lifelong neurological impairments including cerebral palsy, mental retardation, and learning deficits (120–123). Periventricular leukomalacia (PVL) is the predominant form of brain injury and the leading known cause of cerebral palsy and cognitive deficits in preterm infants (124).

Several animal models have been introduced for studies of preterm brain injury. The features, advantages, and disadvantages of each animal model of preterm birth are summarized elsewhere (125). Hypoxia–ischemia mouse, rat, and pig models and chronic hypoxia mouse and rat models have been utilized for the study of disorders in very low birth weight infants. The global mechanism by which perinatal hypoxia alters development is through a delay in the maturation of affected cell types, including astrocytes, oligodendrocytes, and neurons (125). Delays in oligodendrocyte maturation cause delayed myelination, which is typical of preterm white matter damage. By contrast, a proinflammatory status with cytokine imbalance has been reported in PVL (124, 126, 127). In this regard, LPS-induced inflammation mouse, rat, rabbit, and sheep models and an IL-1 β administration mouse model are also useful for studies of preterm brain injury (125, 128).

An especially important characteristic of preterm newborns is that, when they develop systemic inflammation, they have the capacity to prolong the inflammation, resulting in intermittent or sustained systemic inflammation, and thereby increasing the risk of brain damage (129). Intermittent or sustained systemic inflammation contributes more to adverse neurodevelopmental outcomes in preterm infants than shorter duration inflammation (119). Therefore, future studies should address how prolonged systemic inflammation in preterm infants affects immature brains and causes long-lasting neurodevelopmental deficits. Since human babies at 25, 30, and 35 weeks of gestational age correspond to rodent pups at postnatal day 3 (P3), P7, and P10, respectively (125), newborn rodents treated with multiple systemic LPS injections can be a good model of intermittent or sustained systemic inflammation in preterm infants. The mechanism that connects systemic inflammation and microglial activation in preterm brains would be an especially promising avenue of research.

CONCLUSION

The leptomeninges, choroid plexus stroma and epithelium, attachments of choroid plexus, perivascular space, CVOs, and astrocytic endfeet construct the histological architecture that provides a location for intercellular interaction between bone marrow-derived myeloid lineage cells and brain parenchymal cells under non-inflammatory conditions or at early stages

of systemic inflammation during which the BBB is relatively preserved and the brain parenchyma exhibit no necrotic tissue damage. This histological architecture functions as the interface between the brain and the immune system to relay systemic inflammation-induced microenvironmental molecular events to the brain parenchyma. An understanding of how brain parenchymal cells, particularly microglia, respond to signals conveyed in this manner awaits further studies. It is essential that the mechanisms underlying the intercellular interactions among brain parenchyma cells as well as those between immune cells and brain cells be unraveled. Future investigations of these issues would enhance our understanding of the pathogenesis of SAE and of preterm brain injury.

ETHICS STATEMENT

In our experiments described in the present article, all experiments were approved by the Animal Care and Use Committee of the Aichi Human Service Center. All mice were handled in accordance with the guidelines of Aichi Human Service Center and the Guide for the Care and Use of Laboratory Animals (National Academy Press, Washington, DC, USA).

AUTHOR CONTRIBUTIONS

AS designed the article. AS and SH-I contributed to the conception, writing, and editing of the manuscript. AS and SH-I carried out the experiments and analyzed the data in their studies described in the manuscript.

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Role of Inflammation in Human Fatigue: Relevance of Multidimensional Assessments and Potential Neuronal Mechanisms

Bianka Karshikoff^{1,2}, Tina Sundelin^{1,3} and Julie Lasselin^{1,2,4*}

¹Division for Psychology, Department of Clinical Neuroscience, Karolinska Institutet, Solna, Sweden, ²Stress Research Institute, Stockholm University, Stockholm, Sweden, ³Department of Psychology, Stockholm University, Stockholm, Sweden, ⁴Institute of Medical Psychology and Behavioral Immunobiology, University Hospital Essen, Essen, Germany

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Karolinska Institutet, Sweden

*Correspondence:

Julie Lasselin
julie.lasselin@uk-essen.de

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Fatigue is a highly disabling symptom in various medical conditions. While inflammation has been suggested as a potential contributor to the development of fatigue, underlying mechanisms remain poorly understood. In this review, we propose that a better assessment of central fatigue, taking into account its multidimensional features, could help elucidate the role and mechanisms of inflammation in fatigue development. A description of the features of central fatigue is provided, and the current evidence describing the association between inflammation and fatigue in various medical conditions is reviewed. Additionally, the effect of inflammation on specific neuronal processes that may be involved in distinct fatigue dimensions is described. We suggest that the multidimensional aspects of fatigue should be assessed in future studies of inflammation-induced fatigue and that this would benefit the development of effective therapeutic interventions.

Keywords: central fatigue, inflammation, immune system, multidimensional assessments, motivation, ventral striatum, anterior cingulate cortex, insula

INTRODUCTION

Fatigue is a highly disabling symptom that is common in various medical and psychiatric conditions (1–4). In some cases, the origin of fatigue can be explained by alterations in muscle metabolism or the cardiovascular system, but for most clinical populations, such as cancer survivors and patients suffering from multiple sclerosis (MS) or chronic fatigue syndrome/myalgic encephalomyelitis (CFS/ME), fatigue pathophysiology remains hard to establish. In these conditions, inflammation has been hypothesized as a possible contributor (5, 6), based on an extensive literature showing the capacity of inflammatory factors to act on the central nervous system (CNS) and induce behavioral changes, including fatigue (7–9). Furthermore, alterations in inflammatory processes are found in patients suffering from medical conditions also characterized by high rates of fatigue, such as cancer survivors and patients with MS or diabetes (6). However, despite reported associations between fatigue and circulating levels of inflammatory markers in populations of patients suffering from these diseases (10, 11), the specific role and biological mechanisms of inflammation in the development of fatigue symptoms remain elusive. One of the reasons may be that fatigue is rarely assessed from a multidimensional perspective.

FATIGUE: A MULTIDIMENSIONAL PERSPECTIVE

Definitions of Fatigue

The multidimensional aspect of fatigue has been addressed in previous literature. One distinction relates to *peripheral* and *central* fatigue (12), with peripheral fatigue described as “the inability to sustain a specified force output or work rate during exercise,” and central fatigue as “the failure to initiate and/or sustain attentional tasks and physical activities requiring self-motivation” (13). This distinction thus lies mainly in the origin of the feeling, with peripheral fatigue developing from peripheral physiological and neuronal systems (e.g., neuromuscular transmission, muscular metabolism, or the cardiovascular system), whereas central fatigue results from changes in the CNS. Central fatigue is further comprised of several dimensions, namely *physical fatigue*, *mental/cognitive fatigue*, and *motivational changes*. Physical fatigue is characterized by a difficulty in performing physical activities, while mental/cognitive fatigue is described as difficulties concentrating and carrying out cognitive tasks (14). These distinctions reflect the behavioral outputs of central fatigue. For these behaviors, motivational changes appear to be central. Motivational inputs, such as expected rewards and benefits, modulate the effort exerted by the individual in any given situation (15). Hence, fatigue has been suggested to arise when the balance between the energy costs and the expected reward of an action is disrupted (16). Consequently, central fatigue may depend on flawed integrations of motivational inputs and/or energy expenditure (13, 15).

Another important aspect to take into account regarding central fatigue is the distinction between *physiological* and *pathological* fatigue. Biologically, fatigue is first and foremost an adaptive physiological process. It is the reduction of effort, resulting from perceived exertion (appraised by motor and sensory inputs) and motivational factors (15). Fatigue is a signal to rest, and it encourages energy preservation to prevent injuries, which may be beneficial after intense work or sleep loss, or when the bodily resources need to be redirected toward fighting pathogens during an infection (17). Fatigue also helps focus on more energy-efficient actions (16). As such, healthy, normal physiological processes of fatigue are denoted *physiological fatigue* in this review, as opposed to *pathological fatigue*, which is a state where the adaptive function has been lost. Although central fatigue is primarily a feeling, and usually assessed through subjective measurements (e.g., self-report questionnaires), it can also be measured objectively, using physical, cognitive, or motivational tasks.

Taken together, central fatigue appears to be not just “fatigue,” but a complex symptom that comprises several dimensions and concepts (**Figure 1**). In this review, we will focus on the effects of inflammation on central fatigue and illustrate the importance of multidimensional assessments in understanding the pathophysiology of inflammation-induced central fatigue.

Issues to Consider When Studying Fatigue

Fatigue is a highly subjective experience that every human being experiences at some point. This intuitive everyday understanding

of fatigue may complicate formal assessments. A subjective distinction between normal (physiological) but pronounced fatigue *versus* pathological fatigue may be difficult to describe and, as such, the nature and intensity of pathological fatigue may be difficult to understand for relatives and caregivers (18). Furthermore, in everyday speech, fatigue is often used interchangeably with tiredness, which in turn is used as a synonym for sleepiness, i.e., sleep propensity (19, 20). Although fatigue and sleepiness are generally considered different concepts in both research and clinical practice, some assessment scales use sleepiness as a dimension of fatigue (21), and some tasks that induce mental fatigue also cause sleepiness (22, 23). This relationship is further complicated by the concept of tiredness, which may be considered equal to fatigue or as a lesser version thereof (24). In addition, other feelings can also be interpreted as—and overlap with—fatigue, such as boredom (25). Evidently, there is a need for a clear characterization of fatigue, both physiological and pathological. The literature on diseases in which fatigue is one of the main causes of suffering for the patients, such as cancer, may help in this regard. The interdisciplinary workgroup Assessing the Symptoms of Cancer using Patient-Reported Outcomes highlights several characteristics of cancer-related fatigue (26), some of them appearing critical for distinguishing pathological from physiological fatigue. For example, as opposed to physiological fatigue, pathological fatigue is not alleviated by sleep or rest (18) and is not proportional to the degree of activity (27).

Beyond the distinction between physiological and pathological fatigue, the assessment of fatigue should be performed keeping in mind the several dimensions and conflicting or overlapping concepts, as discussed above (**Figure 1**). These dimensions may in fact involve distinct neuronal systems (see Part 3 of the current review). What is called “fatigue” may thus be driven by different underlying mechanisms from one patient to another and from one condition to another. While some self-assessment scales encompass several kinds of fatigue [e.g., the multidimensional fatigue inventory (MFI) (14)], others rely on single or non-specific aspects and, although having clinical relevance, may prevent the understanding of the pathophysiological processes. In addition, while the use of long or intense physical or cognitive tasks assesses fatigue in an objective way, these objective measures do not always correlate with subjective measures, indicating that they may actually assess distinct components (28) and may not be ecologically valid.

Given the large clinical overlap between pain and fatigue (29), the conceptualization of fatigue could be inspired by that of pain. In pain research, both central and peripheral biological components have been identified (30), as well as a fairly well-described neuronal network (31). There are clear mechanistic differences between acute and chronic pain (32), and, depending on the diagnosis, peripheral and central dysfunctions are involved to different degrees (33). However, some components are common for all pain diagnoses (34), and low-grade inflammation has recently been added to this list (35, 36). Following this rationale of pain research, we suggest that there are identifiable biological mechanisms that drive fatigue and that these include peripheral and central components, as well as identifiable neuronal networks. Moreover, the mechanisms may change if the fatigue becomes

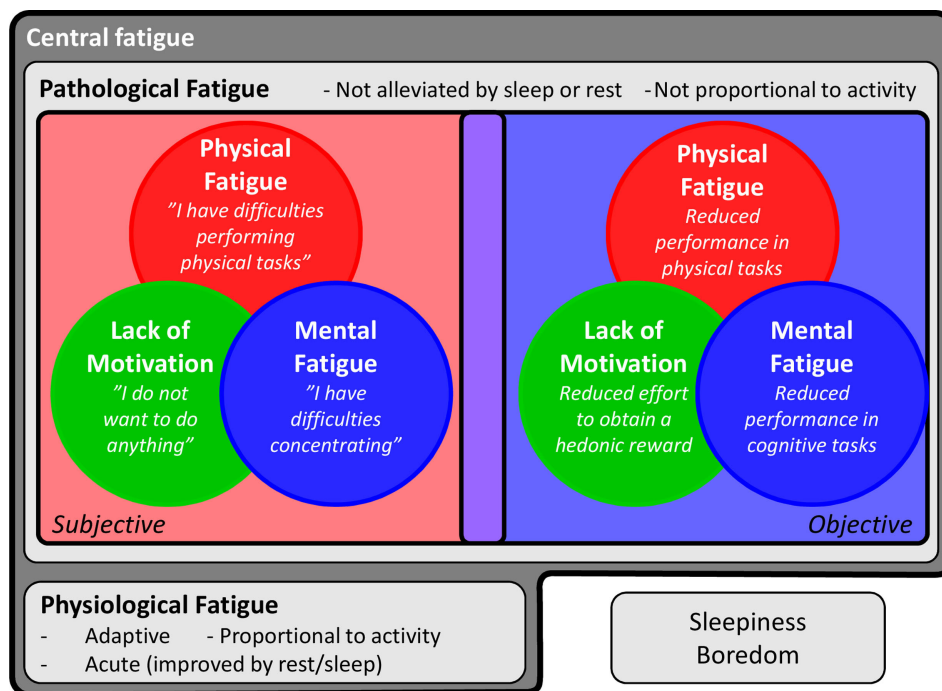


FIGURE 1 | Features of central fatigue. Central fatigue is a complex symptom including several dimensions and concepts. It can be divided into *physiological* fatigue, a signal to rest and encourage energy preservation to prevent injuries, and *pathological* fatigue, when the adaptive function has been lost. Central fatigue is also comprised of several dimensions, namely *physical fatigue*, *mental fatigue*, and *lack of motivation*. These dimensions can be assessed in subjective or objective ways. Finally, the study of fatigue is further complicated by the difficulty in separating fatigue from close but distinct concepts, such as sleepiness and boredom.

chronic and may vary for different conditions. Specifically, we argue that inflammation affects some of the biological systems underlying fatigue and that inflammation may therefore be one of the major driving forces for fatigue.

INFLAMMATION AND FATIGUE

The Activated Immune System Induces Fatigue

During activation of the immune system, immune cells produce pro- and anti-inflammatory cytokines, which are signaling molecules that coordinate the fight against the pathogen. A less-known feature of cytokines is their capacity to act on the CNS, inducing behavioral alterations, including the development of fatigue [for reviews, see Ref. (7, 8)]. The cytokine signal reaches the brain *via* several immune-to-brain pathways, e.g., by a neuronal pathway *via* the vagus nerve or by a humoral pathway *via* brain locations with a weaker blood-brain barrier (37–39). Cytokines in the brain then induce modifications in neurotransmitter and neuroendocrine systems (**Box 1**), along with modifications in brain functions, which lead to behavioral changes.

Although cytokines induce a large array of behavioral changes, including changes in mood and cognitive functions, fatigue is, interestingly, one of the first and most common symptoms associated with an activated immune system (47). This has been demonstrated in patients suffering from cancer or hepatitis

C, who undergo immunotherapy with the pro-inflammatory cytokine interferon- α , which activates the immune system and has neuropsychiatric side effects (48–50). Among these, fatigue develops very rapidly after instauration of the treatment in a large proportion (up to 80%) of patients, while other behavioral alterations, such as depressed mood and cognitive dysfunction, appear later and only in a subpopulation (30–60%) of patients (49, 51). This suggests that fatigue is very sensitive to the effects of cytokines, and underscores the biological connection between fatigue and inflammation.

Clinical Aspects of Inflammation and Fatigue

There is increasing evidence supporting the role of inflammation in fatigue in clinical populations, particularly from cancer and cancer-related fatigue research (11, 52). During cancer treatment, the increase in circulating concentrations of inflammatory markers, such as C-reactive protein (CRP) and interleukin (IL-6), was related to the development of an overall feeling of fatigue (53–55). Inflammation has also been associated with higher levels of post-cancer persistent fatigue. For example, breast cancer survivors who are fatigued, i.e., reporting lower levels of energy or vitality, show signs of activated inflammatory processes with increased concentrations of circulating inflammatory markers, as well as increased *ex vivo* inflammatory response to an immune challenge (56–58). In this population, higher levels of circulating CRP have

BOX 1 | Cytokine effects in the brain.

During the activation of the immune system, immune cells produce cytokines that coordinate the immune response. In addition to their peripheral actions, cytokines are able to signal to the CNS. Several such immune-to-brain communication pathways have been described, including a *neuronal* and a *humoral* pathway. The former refers to the fact that cytokines can activate the vagus nerve at the periphery, which then modulates functions of the brain targets of vagal afferents (38). Cytokines can also activate brain immune cells (microglia) that are located alongside brain vessels where the blood–brain barrier is weaker (e.g., in the circumventricular organs) (39). These cells then produce cytokines locally. The cytokine signal propagates in the brain *via* diffusion, microglial activation, and neuronal projections (37).

A crucial mechanism by which cytokines modulate neuronal functions is through modifications of monoaminergic neurotransmission, specifically by activating enzymes interfering with dopamine and serotonin biosynthesis. One of these enzymes is the GTP-cyclohydrolase 1 (GTP-CH1), which is involved in the production of neopterin. The production of neopterin happens at the expense of the production of tetrahydrobiopterin (BH4), which is an essential cofactor for the biosynthesis of dopamine and serotonin (40, 41). In addition, cytokines activate the indoleamine 2,3-dioxygenase (IDO), the rate-limiting enzyme degrading tryptophan along the kynurenine pathway (7, 42). The degradation of tryptophan reduces its availability for serotonin biosynthesis. By activating GTP-CH1 and IDO, cytokines thus reduce the synthesis of dopamine and serotonin. Cytokines also modulate dopamine- and serotonin-transporter activity, reducing their synaptic availability (43, 44). In addition to neurotransmitter systems, cytokines modulate neuroendocrine systems, such as the hypothalamic–pituitary–adrenal axis, activating the release of corticotropin-releasing hormone, adrenocorticotropic hormone, and cortisol (45, 46).

shown a positive correlation with overall levels of fatigue even after adjusting for several confounders, such as obesity, self-rated health, depression, and insomnia symptoms (59). Although most of these studies used unidimensional assessments of overall fatigue, some have reported an association between inflammation and multidimensional fatigue, probing for different aspects, such as physical or mental fatigue, in cancer patients. These studies indicate that inflammation in cancer patients and survivors may affect particularly the physical rather than the mental aspects of fatigue (60–63). Further investigation, aiming specifically at assessing the role of inflammation in the different dimensions of fatigue, is needed to determine whether inflammation indeed leads mainly to the development of physical fatigue, or whether it also contributes to the cognitive/mental and motivational aspects of fatigue in cancer patients.

The extreme clinical form of fatigue in CFS/ME has been the subject of extensive study and provides a good model for assessing the potential role of inflammation in the development of fatigue (6). CFS/ME is a debilitating multisystem condition primarily defined by a disabling fatigue for more than 6 months, along with several other symptoms, including pain and cognitive changes (64). Due to the nature of the illness, a broad array of fatigue questionnaires are used for this group of patients, measuring several aspects of fatigue (65). One of the key symptoms is “postexertional fatigue” (64), which, interestingly, appears to be somewhat unique for this patient group (65). Although the underlying mechanisms of this disease are complex, a clear immunological component stands out; CFS/ME often appears following an infection, and some of the most promising treatments are immunomodulatory (64, 66). Furthermore, an extensive

literature indicates that patients suffering from CFS/ME exhibit increased systemic production of pro-inflammatory cytokines [e.g., IL-6 or tumor necrosis factor (TNF)- α] and higher CRP at baseline as well as after immune stimulation, compared to non-fatigued individuals (67–72). Altered cytokine production is also associated with the intensity of fatigue symptoms in CFS/ME patients (73, 74). Regarding multidimensionality, CRP concentrations have been found to associate with physical health-related quality of life but not with mental health-related quality of life in a mixed sample of healthy individuals, individuals with high level of fatigue, and patients with CFS/ME (72). Although these measures of health-related quality of life do not specifically assess fatigue, this study highlights the fact that inflammation may be only related to certain dimensions of symptoms or symptom clusters. This has also been indicated in other clinical conditions, such as type 2 diabetes. Type 2 diabetes is characterized by low-grade but chronically increased concentrations of inflammatory markers, found to be closely associated with mental fatigue and lack of motivation, but not with physical fatigue (10). In other patient groups, inflammation has been found to relate to several dimensions of fatigue, both physical and mental. This is the case for patients suffering from MS, in which inflammation correlates with both physical and cognitive dimensions of fatigue, as well as with sleepiness (75).

Taken together, inflammation may be a key player in the development of pathological fatigue. However, the few studies assessing the role of inflammation in fatigue using a multidimensional perspective indicate that inflammation may not always relate to all dimensions of fatigue. In patient groups with long-term fatigue and comorbidity, other factors may thus be of greater importance for some aspects. Importantly, we do not advocate an “inflammation-specific type of fatigue,” but argue that the fatigue dimensions that are affected by inflammation may vary in different medical conditions. This is of high importance when considering the development of anti-inflammatory therapeutic interventions to improve fatigue in patients. Pharmacological treatments aiming at blocking the actions of cytokines, such as inhibitors of TNF- α , have been found to clinically reduce fatigue in patients suffering from rheumatoid arthritis or psoriasis (76, 77). However, if inflammation relates only to a specific aspect of fatigue in a certain population, the use of cytokine inhibitors may only improve certain types of fatigue. For instance, medication with a monoclonal antibody against IL-1 β (XOMA052) was found to affect physical, but not cognitive fatigue in type 2 diabetes (78). A better understanding of the effect of inflammation on the multidimensional aspects of fatigue in medical conditions is therefore essential for long-term clinical applications.

Inflammation and Fatigue in the General Population

Inflammation does not only relate to fatigue in clinical populations, but there is also a connection between inflammatory activity and fatigue in the healthy population. Inflammation, as measured with CRP levels, has been found to predict the development of fatigue in healthy subjects 5 years later, even after adjusting for several confounders (79). In addition, a recent study has shown

that CRP concentrations in the general population are associated with higher fatigue and reduced sleep quality, but not altered mood or concentration difficulties (80). An earlier study using a multidimensional assessment of fatigue in healthy individuals, however, contradicts these findings, showing that depressive symptoms and adiposity were better predictors of overall and physical fatigue than inflammation (81). Similar results were found in older individuals, for which the association of circulating concentrations of CRP and IL-6 with overall and physical fatigue was no longer significant when adjusting for depressive symptoms or adiposity (82). These results may at least partially be due to sex differences, as the authors also reported a significant relationship, independently of depressive symptoms and adiposity, between CRP levels and fatigue intensity in women but not in men (83).

Inflammation thus seems to contribute to the development of fatigue even in the general population, but a multidimensional assessment is generally lacking. This is unfortunate given that it may prevent the understanding of the pathophysiology of fatigue. Indeed, the different dimensions of fatigue may involve distinct underlying neurological processes. One illustrating example relies on motivational changes, which may drive, at least partially, inflammation-induced fatigue (84). Decreased motivation results from specific alterations in reward-related neuronal processes, involving notably the mesolimbic dopamine pathway (85).

POTENTIAL NEURONAL MECHANISMS UNDERLYING DIMENSIONS OF INFLAMMATORY-INDUCED CENTRAL FATIGUE

As described in **Box 1**, during the activation of the immune system, the inflammatory cytokine signals reach the brain (38, 39), inducing changes in neurotransmitter and neuroendocrine systems, and leading to behavioral changes (7, 8). For example, cytokines can inhibit the synthesis of neurotransmitters, such as dopamine or serotonin, by activating specific enzymes involved in the rate-limiting steps of their biosynthesis (86, 87). These alterations in neurotransmitter systems ultimately lead to modifications in neuronal functions, which in turn induce behavioral changes collectively called *sickness behavior*. Sickness behavior includes fatigue, reduced activity, altered mood state, changes in cognitive functions, and reduced appetite. Sickness behavior is an adaptive process allowing the body to rest and to redirect energy toward fighting infections (17, 88). Although most of the effects of cytokines on the CNS have been demonstrated with high levels of circulating cytokines (e.g., after an immune challenge or during immunotherapy), evidence also suggests that low-grade levels are enough to affect the brain (89, 90). Interestingly, the specific modifications of CNS functions during immune system activation can help infer some mechanisms that likely underlie inflammation-induced central fatigue. Notably, imaging studies of immune challenges highlight changes in activation of the anterior cingulate cortex (ACC), the anterior insula, and the ventral striatum (87, 91). As these areas have also been associated

with fatigue in several medical conditions, they seem likely to underlie inflammation-induced fatigue symptoms (92). Here, we take this one step further and propose that specific functional brain alterations induced by inflammation may contribute to the development of the different dimensions of fatigue.

The Basal Ganglia

Given that motivation is a core feature of fatigue and that inflammation has been shown to modulate reward-related processes (88, 93, 94), it is possible that these reward-related processes are involved in the effect of inflammation on fatigue (84). The mesolimbic dopamine pathway, linking the ventral tegmental area to the nucleus accumbens (in the ventral striatum), is essential in the modulation of motivation (85, 95) and particularly in effort-related motivational behaviors (96). This “non-motor part” of the basal ganglia has been suggested as a critical mechanism for the development of central fatigue (13, 97). Altered dopamine processes in the ventral striatum can lead to an effort-reward imbalance, with increased perception of energy costs of actions and/or decreased expectation of reward or benefits (16). This can lead to the feeling of physical and/or mental fatigue, although the underlying issue is a reduced motivation to perform physical or cognitive tasks (98). Additionally, even though fatigue research has focused mainly on the motivation pathway of the basal ganglia, the motor pathway may be involved as well. Decreased volume and activation of the putamen, caudate, and pallidum have been described in fatigued patients with MS or CFS, and are associated with the intensity of fatigue symptoms (99, 100).

Several lines of research indicate that inflammation may induce the development of fatigue, specifically through reduced motivation *via* alterations in basal ganglia functions. Cytokines are known to affect dopamine function (see **Box 1**), which leads to modifications in basal ganglia activity, such as the mesolimbic dopamine pathway. A reduced activation of the ventral striatum in response to hedonic reward has indeed been observed after an immune challenge (93, 101). This functional change has been suggested to underlie the development of cytokine-induced fatigue (102, 103). Furthermore, fatigue, but also psychomotor slowing, that develops after the instauration of immunotherapy appears to relate to modifications in dopamine function (while mood and cognitive dimensions rather relate to serotonin function) (49–51, 104). After the commencement of cytokine therapy, patients also exhibit increased glucose metabolism in the basal ganglia, which is associated with symptoms of fatigue and reduced motivation as assessed with the MFI (105, 106). In addition, the reduction of ventral striatal activity in response to reward observed during immunotherapy is associated with reduced motivation, reduced activity, as well as depressive symptoms (101). Immunotherapy-induced physical fatigue, measured as decreased energy, was found to relate to increased basal activity both in the putamen and the ventral striatum (107), but motivational changes were not assessed in this study. Additionally, a recent study nicely illustrates the specific contribution of the ventral striatum in cytokine-induced fatigue (108). In this study, very early ventral striatal alterations induced by immunotherapy (4 h after the initiation of immunotherapy) significantly predicted the later development of fatigue (4 weeks follow-up). Importantly, these changes in striatal

function did not predict mood symptoms, which supports the idea that inflammatory effects on the brain may be separated into distinct circuits that underlie the different parts of sickness behavior, some of which drive fatigue specifically. Nevertheless, fatigue was not assessed in a multidimensionality perspective in this study, and specific changes in motivation were not evaluated.

These studies highlight the potential contribution of the basal ganglia, particularly the mesolimbic pathway, in inflammation-induced fatigue. While these studies have been conducted in conditions of high-level activation of the immune system, some data on older adults suggest that inflammation at a low-grade state is sufficient to induce alterations in the dopamine system, contributing to the development of fatigue (41). However, although some of these studies have used multidimensional assessments of fatigue, including reduced motivation, those assessing the effect of inflammation on basal ganglia changes usually measure either fatigue or motivational changes, but rarely the two together.

The ACC

The ACC has been implicated in inflammation-driven processes in several studies (105, 109, 110), and we suggest that this area could be related to the cognitive aspects of fatigue. The ACC, in particular the dorsal part, is involved in conflict monitoring (111–113) and in cognitive control (114). Activation of the dorsal ACC seems to signal the adjustment of cognitive processes according to the difficulty or cognitive demand of the task (115). Interestingly, it has been suggested that the feeling of cognitive/mental fatigue may arise from an increased cerebral effort to maintain a satisfactory performance (116). An increased activation of the ACC during a motor or mental task has been shown in fatigued patients with CFS or MS (117, 118) and was associated with a feeling of having to exert more effort (118). Thus, it is possible that the stronger activation of the ACC signals a need for increased cognitive processing, leading to a feeling of mental fatigue.

Inflammation-induced sickness may represent a more demanding mental state for an individual than full health does, as indicated by a decline in cognitive abilities during immune activation (119). Altered activation of the ACC, mostly an increase in the dorsal part, has been repeatedly reported during activation of the immune system (105, 109, 110, 120, 121). Importantly, the dorsal ACC was the structure most strongly activated during an attentional task in patients treated with immunotherapy, in comparison to control subjects (109). This activation also correlated with number of errors, in line with the involvement of the dorsal ACC in conflict monitoring. Furthermore, inflammation-induced fatigue during a more acute model (typhoid vaccination) was found to significantly correlate with the activation of the ACC during a mental conflicting task (the Stroop task) (122). This was, however, not the case for those feeling fatigued after placebo, suggesting a specific mechanism of inflammation on ACC functions in the development of fatigue.

The Insula

There is a growing interest in the potential role of the insular cortex in inflammation-induced fatigue. This brain area is considered a main hub for the perception of the physiological

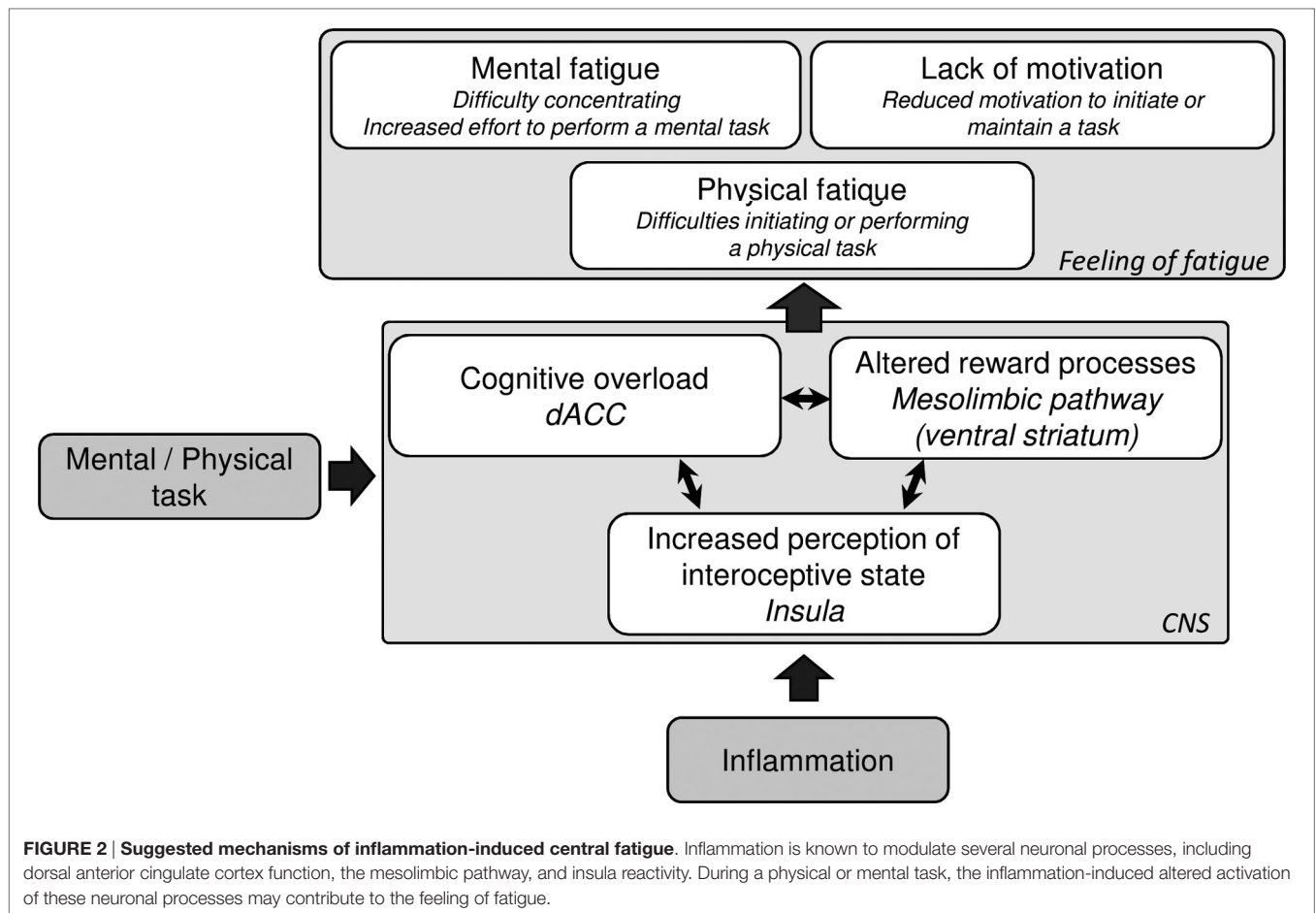
condition of the body, so-called interoceptive signals, and it has been suggested as the central structure for “human awareness” (123, 124). Speculatively, a tiresome task would require insular involvement for the brain to interpret the associated bodily signals, and the behavioral output to restore homeostasis and promote rest would be the feeling of fatigue. An increased responsiveness of the insula to interoceptive signals would, therefore, make individuals more prone to feeling fatigued. Interestingly, it has repeatedly been shown that inflammation increases insular activity (121, 122, 125–127). Two studies even show a relationship between inflammation-induced insular function and fatigue development (122, 126). In addition, patients with MS, a condition characterized by both alterations of inflammatory processes and fatigue, exhibit increased activation of the insula during a motor task (128). To speculate further, inflammation may thus induce increased sensitivity to interoceptive signals, through stronger insula reactivity, leading to a more rapid development of an overall feeling of fatigue when performing tasks.

Other Central Processes

It is not our intention to reduce fatigue processes to the three brain structures above, and additional brain structures could very well contribute to fatigue in the situation of immune activation. For instance, the self-regulatory and cognitive functions of the pre-frontal cortices are likely to play an important role in the modulation of fatigue (129, 130). Nevertheless, our aim was to highlight that different neuronal functions may underlie different dimensions of fatigue and that more (multidimensional) studies are needed to comprehend the involvement of inflammation in its pathogenesis.

In addition, beyond the functions of specific brain areas, changes in the connections between structures may also underlie the development of fatigue (131–133). This research is still in its infancy, but bears great potential for understanding potential mechanisms. Inflammation has been shown to affect intrinsic connectivity (134–136) and, for instance, the connectivity between the insula and mid-cingulate cortex seems associated with the inflammation-induced state of malaise and discomfort, in line with the interoceptive role of insula (136). Although this has not yet been studied in relation to fatigue, it is probable that altered connections between structures, in addition to specific structural changes, also contribute to the development of fatigue (97, 137).

In summary, inflammation appears to induce changes in neuronal functions that in turn may contribute to the development of fatigue (Figure 2). Although the specific involvement of the cerebral structures for the different dimensions of fatigue remains to be elucidated, we argue that a higher cognitive load during inflammation could lead to a feeling of mental fatigue and depend on changes in ACC function. The mesolimbic reward system on the other hand, may be involved in the dimension of fatigue that relates to lack of motivation, a feature that may be particularly prominent in inflammation-induced fatigue. Finally, higher sensitivity to interoceptive signals may induce an overall feeling of fatigue. While inflammation may be involved in all these processes, it is also possible that, in some medical conditions or



patient subpopulations, inflammation contributes to only one or some of these processes. It is therefore important to characterize the specific dimensions of fatigue that develop in patient populations, and assess the role of inflammation. For instance, some aspects of fatigue may be derived from sleep alterations or changes in hormonal regulation in clinical populations, e.g., of insulin or cortisol, which can modulate brain functions including those in the above-mentioned areas (138–140).

CONCLUDING REMARKS AND FUTURE CONSIDERATIONS

In this review, we have highlighted the potential role of inflammation in the development of pathological central fatigue. Importantly, we wanted to illustrate the need for multidimensional assessments of fatigue when assessing the role of inflammation, given that fatigue contains distinct features that may be explained by separate central mechanisms and may be specific to different medical conditions. Studying fatigue using a multidimensional perspective also appears highly relevant for the development of therapeutic interventions that target inflammation in order to improve fatigue. In cases where inflammation contributes to only some aspects of fatigue, the use of anti-inflammatory therapies may not be sufficient to improve the feeling of fatigue.

It is therefore important to disentangle the dimensions of fatigue if we are to understand the pathophysiological role of inflammation in this symptom. While the use of single, general measures of fatigue is sometimes preferable, depending on the researcher's or clinician's need (27), the choice of the fatigue measurement(s) should be carefully considered with regard to the study aims (141). This is especially true since no gold-standard measure exists at this point. However, some recommendations can be made for when the aim is to understand the underlying pathophysiological processes. Several self-report scales of multidimensional fatigue are available, such as the MFI (14), the Swedish Occupational Fatigue Inventory (SOFI) (21), the Checklist Individual Strength (CIS) (142), and the Multidimensional Fatigue Symptoms Inventory (MFSI) (143). It is also important to take into account the time span of fatigue. Hence, while one may assess the feeling of fatigue over a long period of time when referring to pathological fatigue (e.g., 1 or 2 weeks as assessed with the MFI, CIS, or MFSI), measuring acute changes in fatigue when assessing the effects of inflammation is also crucial. This can be done by repeated assessments of the level of fatigue that the subject feels at the time of scale completion, as measured with the SOFI (which, however, lacks a mental fatigue dimension) or visual analog scales, such as the Visual Analogue Scale for Fatigue (VAS-F) (144), which only focuses on physical

fatigue. The use of an acute measurement of fatigue also allows for evaluating subjective fatigue induced by physical, mental, or motivational tasks. Using a fatigue-induced task can help define fatigue in a more precise way than when relying solely on reports that pertain to the past few weeks. Needless to say, the development of new scales would be beneficial to the field. These should include both chronic and acute fatigue, as well as the multidimensional features. In the meantime, we suggest using a combination of different scales. In addition, self-report scales could be combined with objective measures of fatigue, such as reduction of performance during a physical or mental task. Effort-related reward tasks, such as the Effort Expenditure for Reward Task (EEfRT) (145), in which subjects receive a monetary reward for effort, may also help in understanding the role of motivational changes in fatigue (146). Nevertheless, it is preferable to combine these objective assessments of fatigue with subjective measures, given that objective fatigue is not always associated with subjective reports of fatigue (28), and that fatigue is first and foremost a subjective experience.

In conclusion, although fatigue is increasingly taken into account by clinicians, the study of this symptom remains limited by being restricted to overall fatigue, which, as highlighted in this review, may encompass many different mechanisms. While inflammation may be involved in the development of fatigue, the specific underlying mechanisms remain poorly understood, perhaps partly because the different dimensions of fatigue are too rarely explored. The mechanisms underlying other inflammation-induced neuropsychiatric symptoms have been inferred thanks

to multidimensional assessments (51), and this strategy should be pursued when studying inflammation-induced fatigue as well. Fatigue is a critical and highly disabling symptom for many patient groups and individuals. We argue for the need of adequate multidimensional assessments in order to increase the understanding of the mechanisms underlying inflammation-induced fatigue, as well as for the development of effective therapeutic interventions.

AUTHOR CONTRIBUTIONS

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Neuroimmune Interaction in the Regulation of Peripheral Opioid-Mediated Analgesia in Inflammation

Susan Hua^{1,2*}

¹School of Biomedical Sciences and Pharmacy, University of Newcastle, Callaghan, NSW, Australia, ²Hunter Medical Research Institute, New Lambton Heights, NSW, Australia

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Edited by:

Sanae Hasegawa-Ishii,
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*Correspondence:

Susan Hua
susan.hua@newcastle.edu.au

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Peripheral immune cell-mediated analgesia in inflammation is an important endogenous mechanism of pain control. Opioid receptors localized on peripheral sensory nerve terminals are activated by endogenous opioid peptides released from immune cells to produce significant analgesia. Following transendothelial migration of opioid-containing leukocytes into peripheral sites of inflammation, opioid peptides are released into a harsh milieu associated with an increase in temperature, low pH, and high proteolytic activity. Together, this microenvironment has been suggested to increase the activity of opioid peptide metabolism. Therefore, the proximity of immune cells and nerve fibers may be essential to produce adequate analgesic effects. Close associations between opioid-containing immune cells and peripheral nerve terminals have been observed. However, it is not yet determined whether these immune cells actually form synaptic-like contacts with peripheral sensory terminals and/or whether they secrete opioids in a paracrine manner. This review will provide novel insight into the peripheral mechanisms of immune-derived analgesia in inflammation, in particular, the importance of direct interactions between immune cells and the peripheral nervous system.

Keywords: peripheral nervous system, immune cells, neuroimmune, inflammation, pain, opioids

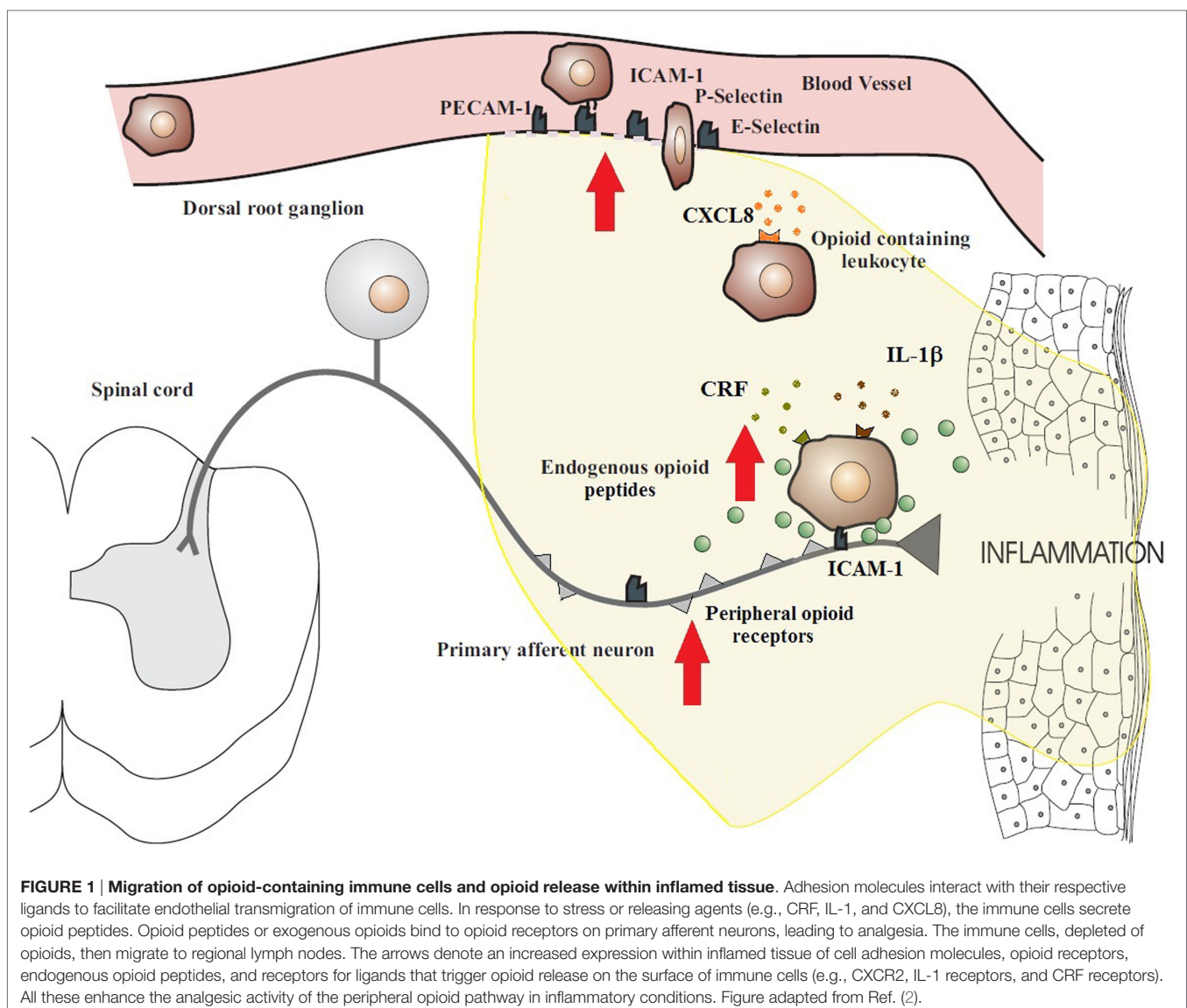
INTRODUCTION

Peripheral opioid mechanisms of endogenous pain control are potent and of clinical relevance. In addition to animal studies, a sizeable body of clinical literature has clearly shown that opioid receptors localized on peripheral sensory nerve terminals can be activated by both exogenous opioid agonists, as well as by endogenous opioid peptides expressed in immune cells, to produce significant analgesia (1–8). This local opioid-mediated analgesia is devoid of the central side effects of systemic opioid administration (e.g., respiratory depression, sedation, and nausea) and has a relative lack of tolerance after repeated administration of peripheral opioid agonists in inflamed tissue (1, 9, 10). In the early stage of inflammation, granulocytes (esp. neutrophils) are the major opioid-containing leukocyte, whereas at later stages of inflammation, monocytes/macrophages and lymphocytes (esp. activated T- and B-cells) predominate (11–14). Inflammation increases the expression of opioid peptides as well as their mRNA transcripts encoding their precursor proteins within these immune cells (14, 15), with β -endorphin (β -END) from pro-opiomelanocortin (POMC) being the most prominent (7, 16, 17). Studies to date suggest that only a finite number of the total immune cell population actually produce opioid peptides and home to lymph nodes. This is supported by the observation that β -END and POMC mRNA were less abundant in circulating

lymphocytes than in those in lymph nodes (14, 18). Ongoing research is focused on differentiating this sub-population of opioid-producing leukocytes for the design of novel targeted therapies. Of even greater interest is what happens once the immune cells enter the inflamed tissue, especially the interaction between the immune cells and peripheral sensory nerve fibers. The proximity of immune cells and nerve fibers may be essential in inflammation, as the overall increased metabolic environment within inflamed tissue increases the activity of opioid peptide metabolism (19–22). As a result, successful pain control may rely on the immune system being even more selective about the location at which opioid peptides are released for efficient and effective pain control (21, 23, 24). This review will provide novel insight into the peripheral mechanisms of immune-derived analgesia in inflammation, in particular, the importance of direct interactions between immune cells and the peripheral nervous system.

MECHANISMS OF PERIPHERAL OPIOID-MEDIATED ANALGESIA

With the duration of inflammation, the number of infiltrating immune cells as well as total opioid peptide content increases steadily at the site of tissue injury. Leukocyte homing, in general, is a multistep process involving the sequential activation of various adhesion molecules located on immune cells and on the vascular endothelium (1, 2) (**Figure 1**). Initially, circulating leukocytes tether and roll along the vascular endothelial cell wall, a process mediated by selectins on leukocytes (L-selectin) and endothelial cells (P- and E-selectin) (6, 25). Leukocytes are then activated by chemokines released from inflammatory cells and presented on the luminal surface of the endothelium (26, 27). This subsequently leads to upregulation and increased avidity of leukocyte integrins, in particular CD49d/CD29 and CD18, which mediate the firm adhesion of leukocytes to endothelial cells by



interacting with members of the immunoglobulin superfamily (e.g., ICAM-1) (26, 27). Thereafter, the cells transigrate through the endothelium directed predominantly by PECAM-1 expressed on endothelial cells at intercellular junctions and are then directed to the sites of inflammation. All these molecules are constitutively expressed and are upregulated in inflammation, except L-selectin, which is rapidly shed upon activation (26, 28). The relatively low expression of L-selectin on opioid-containing leukocytes is most likely due to its shedding required for leukocyte extravasation (26, 28).

In peripheral inflammatory states, opioid-containing immune cells “home” to the inflamed tissue where they release opioid peptides and then travel to local lymph nodes (1, 16). In particular, CD4+ T cells are able to recirculate from the blood, through tissues, into the lymphatic system, and back to the blood. These immune cells exit from the vascular compartment *via* specialized high endothelial venules (HEV) in lymphoid organs. Lymphocyte traffic across the HEV may increase substantially within 3 h following an immune response and by as much as 10-fold over the first 48 h of the response (29, 30). Multiple endogenous factors are able to trigger the release of opioid peptides from immune cells, including environmental stimuli [e.g., stress-induced release of

sympathetic neuron-derived noradrenaline (NA)] (31) and local inflammatory factors [e.g., corticotropin-releasing factor (CRF), interleukin-1 β (IL-1 β), and chemokine CXCL8 (also known as IL-8)] (Figure 1) (2, 18, 21). It has been observed that inflammation upregulates the production of endogenous CRF, IL-1 β , and CXCL8 in inflamed tissue as well as the expression of their respective receptors on leukocytes (32). In addition, adrenergic α 1, β 2, and to a lesser degree, α 2 receptors are expressed on β -END-containing inflammatory cells located in close proximity to sympathetic nerve fibers in inflamed paws (31). It should be noted that these factors can also affect other cells at the site of tissue injury to further promote inflammation, thus contributing to the dynamic pro-inflammatory and anti-inflammatory balance. Opioid release from immune cells has been demonstrated to be calcium dependent, which is consistent with a regulated pathway of release from secretory vesicles, similar to neurons and endocrine cells (16, 33). Subsequently, the opioid peptides penetrate the damaged perineurial sheath and activate opioid receptors on peripheral terminals of sensory neurons to produce endogenous analgesia (6, 8) (Figure 2). Increasing studies have also suggested an anti-inflammatory role for peripheral opioids (4, 34). Several mechanisms have been postulated, including inhibition of NA,

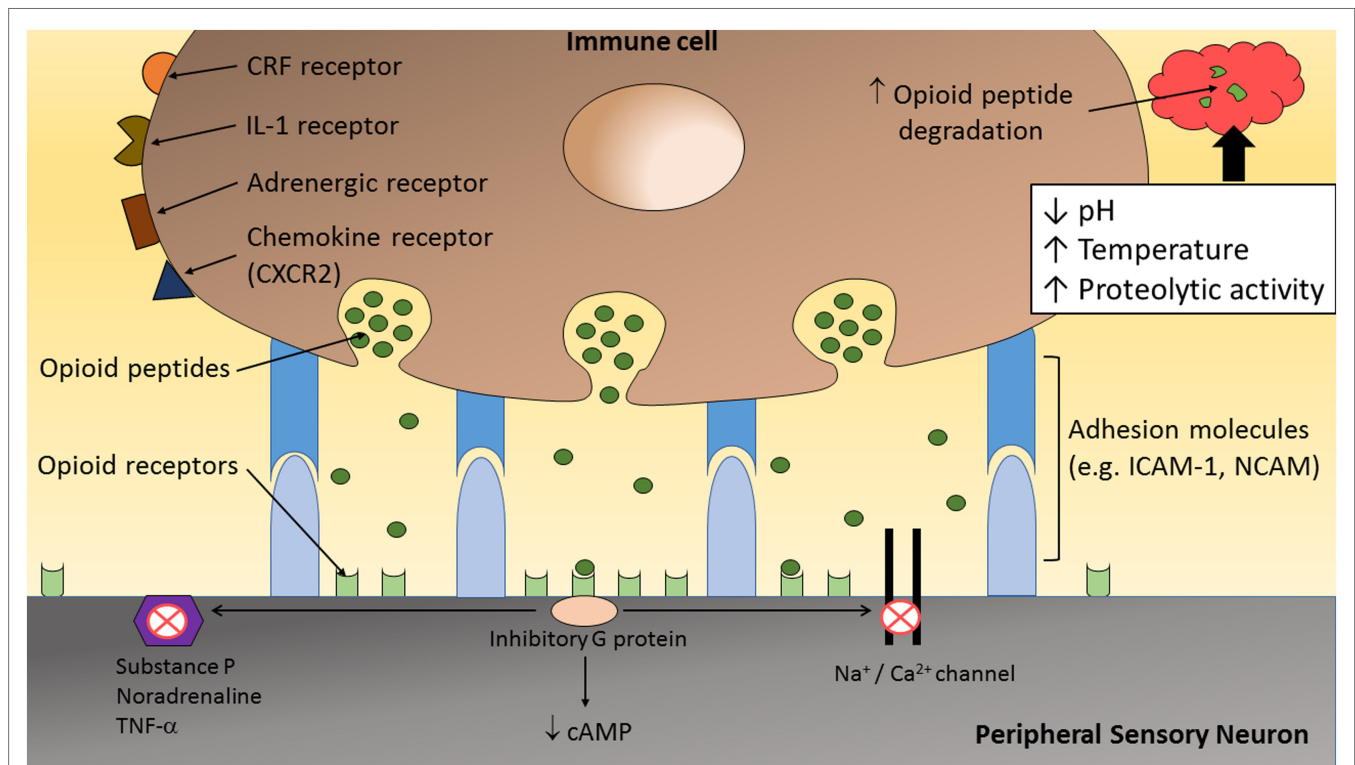


FIGURE 2 | The inflammatory milieu is associated with an increase in temperature, low pH, and high proteolytic activity, which together has been suggested to increase the degradation of opioid peptides (1, 23). Therefore, direct adhesion between opioid-containing immune cells and peripheral sensory neurons, *via* adhesion molecules (e.g., ICAM-1 and/or NCAM), may be necessary to release opioid peptides within the effective range of peripheral opioid receptors to produce adequate analgesia. On activation by opioid agonists, opioid receptors undergo conformational changes allowing intracellular coupling of inhibitory G proteins to the C terminus of opioid receptors. This leads to inhibition of calcium and/or sodium channels and a decrease in the level of neuronal cAMP. In addition, opioids reduce the excitability of nociceptors, the propagation of action potentials, and the release of excitatory and/or pro-inflammatory factors (e.g., substance P, TNF- α , and NA) from peripheral sensory neurons.

substance P, and TNF- α release from neuronal cells (35, 36). The function of NA in inflammation is contested with evidence being provided for both a positive role (37) and a negative role (38).

INFLAMMATION ENHANCES DEGRADATION OF OPIOID PEPTIDES

The precise interaction occurring between opioid-containing immune cells and peripheral sensory neurons is only beginning to be elucidated. Inflamed tissues have been shown to have increased metabolic breakdown rates for opioid peptides (19–22). Hence, it is likely that, for adequate analgesia to occur, direct interaction between these cells are required to allow the release of opioid peptides in close proximity to peripheral opioid receptors on sensory neurons (**Figure 2**). Following tissue injury, the extracellular matrix comprises a myriad of inflammatory mediators (e.g., hydrogen ions, cytokines, and chemokines) and enzymes (39, 40). Activated leukocytes may modify the composition of the extracellular matrix by secreting cytokines and degradative enzymes such as matrix metalloproteinases (MMPs), heparanases, and serine proteases (40). Therefore, following the transendothelial migration of opioid-containing leukocytes into peripheral sites of inflammation, opioid peptides are released into a harsh milieu associated with an increase in temperature (21, 39, 41), low pH (21, 39, 41, 42), and high proteolytic activity (19, 20, 22, 40, 43). Together, this microenvironment has been suggested to increase β -END degradation, which is supported by the short-lasting antinociceptive effect following a single local injection of an exogenous opioid peptide (21).

Endogenous opioid peptides are rapidly degraded by human peripheral blood proteases, giving a half-life of approximately 5 min for enkephalins and 40 min for β -END (19, 22, 24). However, within peripheral inflamed tissue, opioid peptides are exposed to hydrolysis by additional groups of enzymes, including plasma soluble peptidases, peptidases present in the membrane of immune cells, and peptidases released by immune cells (19). In addition, peptidases bound to the extracellular surface of neurons (44) and those associated with opioid receptors (45) degrade opioids in their microenvironment. Consequently, pro-enkephalin (PENK)-derived peptides are very susceptible to proteolytic action resulting in short-lasting central and peripheral antinociceptive actions (19, 46). Endothelial cell enzymes have also been shown to degrade human β -END into various peptide fragments (43). Administration of inhibitors of enzymatic degradation of these peptides, including enkephalinase and amino-peptidase inhibitors, has been shown to augment the duration of action of opioid peptides (46).

Furthermore, tissue acidosis may be responsible for the enhanced activity of various enzymes and the breakdown of substances, including denaturation of proteins and peptides (47). In fact, average proton concentrations as acidic as pH 5.5 have been observed in inflamed tissues, although this may, however, underestimate the true degree of tissue acidification in the inflammatory foci (39). Several inflammatory processes are responsible for this localized acidosis, including the release of various chemical mediators of pain such as hydrogen ions and the local production

of lactic acid (39, 42). In addition, activated neutrophils have been suggested to generate large amount of metabolic acids (e.g., succinic, butyric, propionic, hydrobromic, and hypochlorous acid) (40, 42, 48) and to release an estimated 150 mmol H⁺ ions/liter cells (42). These immune cells further enhance the extrusion of acid and thus contribute to tissue acidosis through the activation of various H⁺ transport processes, in order to maintain their cytosolic pH within physiological limits (42).

Taken together, the analgesic effects of endogenous opioid peptides may depend considerably on their site of secretion from immune cells. It is suspected that the “clouds” of endogenous opioid peptides released from immune cells within inflamed tissues are rapidly surrounded and hydrolyzed by peptidases, resulting in negligible peripheral antinociception (1). Therefore, the release of opioid peptides from immune cells in close apposition to peripheral sensory neurons would maximize the potential for analgesic effects (1, 23) (**Figure 2**).

INTERACTION BETWEEN IMMUNE CELLS AND NEURONS IN PERIPHERAL ANALGESIA

Increasing evidence indicates that the nervous and immune systems are not disparate entities. Immune cells have been shown to interact directly with neurons, with comparisons having been made in the literature between neuronal and immunological synapses (49, 50). Previous studies have reported the innervation of lymphoid organs (51–53), skin (54, 55), eye (56), respiratory tract (57–59), gastrointestinal tract (60–64), liver (65), and the CNS (66, 67) by nerve terminals directly adhering or in close proximity to leukocytes. In line with these findings, close association between peripheral nerves and opioid-containing immune cells have previously been observed (1, 23, 68). *In vitro* studies have demonstrated consistent alliance between lymphocytes containing opioids and cultured DRG nerves (23), while *in vivo* studies have observed this same phenomenon in peripheral inflamed tissues with primary afferent nerves (68). It is plausible that this firm adherence between immune cells and primary afferent nerve fibers may have a functional role in releasing opioid peptides close to opioid receptors within inflamed tissue to provide adequate analgesia.

Anatomical and Functional Neuroimmune Interactions

Anatomical and functional relationships between nerve fibers and immune cells have been highlighted in the literature (49, 50, 69–72). Reports have described the non-random spatial association and bidirectional communication of nerves and immune cells in a variety of tissues in which actual membrane–membrane contacts have been observed (51–53, 57, 58, 60, 65). This concept of a dialog between the immune and sensory nervous system has been based on three observations. First, nerve terminals have been found in direct contact with immune cells (50, 71, 73). Anatomical studies have reported origin, pattern of distribution, and targets of nerve fiber populations supplying lymphoid organs

(51–53, 74, 75). For example, electron microscopy has revealed direct contact between noradrenergic nerve terminals and lymphocytes in the spleen (53, 74, 76). The nerve endings were observed forming long smooth zones of contact with lymphocyte plasma membranes, creating a small cleft of only 6 nm (50, 76). It should be noted that gap junctions are generally 2 nm and classical synapses are 20 nm in width (50). In addition, many of the terminals were found to indent into a lymphocyte, and the apposing membranes were often prominent or slightly thickened (76). These contacts have been suggested to promote bidirectional and chemically mediated transmission between nerves and immune cells with transmitter release and postsynaptic receptor activation (52, 53, 74, 75). In particular, cytokines and opioids released by macrophages and lymphocytes can alter neural NA release from presynaptic varicosities (53, 76). Thus, the likelihood of both local paracrine secretion of NA into the splenic parenchyma and true neuroimmune synapses with lymphocytes, exists, which may represent a key link between the autonomic and immune system.

Second, both organ systems share common receptors and ligands (50, 71, 73). In several studies, significant concentrations of binding sites for a number of neurotransmitters and peptides have been identified on the surface of immune cells and neurons. These receptors have been shown to respond *in vivo* and/or *in vitro* to the neural substances, and their manipulation can alter immune responses (71, 72, 75). This direct influence of the nervous system on cellular immune response is evident in the liver given that lymphocytes and other immune cells expressing opioid receptors were regularly found in close apposition to nerve fibers containing dynorphin (a high affinity endogenous kappa opioid receptor ligand) in the liver of both mice and rats (65). This neuroimmune interaction was suggested to have functional roles in inflammation.

Finally, mutual ligand–receptor interactions lead to significant changes in cellular functions in both systems (21, 39, 41). For example, interactions between nerves and inflammatory cells have been shown to lead to a number of important physiological responses in the lungs (57–59, 65, 77). Studies have suggested that contact between eosinophils and cholinergic nerves may be responsible for vagal hyperreactivity by triggering eosinophil degranulation and a subsequent increase in acetylcholine release from the nerves (57, 58). This direct adherence was dependent on the interaction between the eosinophil integrins CD11/18 and VLA-4 with the neuronal adhesion molecules ICAM-1 and VCAM-1 (57, 58). These cell adhesion molecules are expressed in response to cytokines present in the inflammatory milieu, with inhibition of either adhesion molecule preventing eosinophil-nerve binding (57, 58).

This intimate association of immune cells and neurons in various tissues has been proposed as one of the anatomical bases of communication between the immune and the nervous systems. However, the specificity of anatomical associations between these interactions is beginning to be understood. These synaptic-like contacts may provide the transmitter, or specifically opioid peptides, in even higher concentration for a more immediate effect than is available at a distance (49, 50, 70, 71). Therefore, a functional role following direct interaction between opioid-containing immune cells and peripheral sensory neurons may be

possible (23) with the enhanced recruitment of lymphocytes, the upregulation of opioid peptides, opioid receptors, and cell adhesion molecules in inflammatory conditions (1, 6).

Establishing Functional Connectivity between Neuronal and Immune Cells

Close associations between opioid-containing immune cells and peripheral nerve terminals have been observed (23, 68). However, it is not yet determined whether these immune cells actually form synaptic-like contacts with peripheral sensory terminals and/or whether they secrete opioids in a paracrine manner. In order to substantiate productive interactions at a cellular level between peripheral nerves and the immune system, evidence needs to be accumulated that the criteria established for synaptic connectivity are met (49, 50, 68, 70, 78).

A synapse is a stable adhesive junction between two cells across which information is relayed by directed secretion. Specific qualities of a synapse, irrespective of the cells involved, have previously been described (78). Synapses are utilized in both the nervous and immune systems to directly convey and transduce highly controlled secretory signals between their constituent cell populations. The neuroimmune synapse refers to specialized zones between neurons and immune cells or antigen-presenting cells (APC), and, therefore, can be thought of as a hybrid structure between neuronal and immunological synapses (49, 70, 78). Reports in the literature have clearly established that the immune and nervous systems share common mediators (50, 71, 73). Not only can cells in both systems synthesize and release these mediators but also they both can show physiological responses based on the presence of specific receptors (49, 70, 78). For example, immune cell function within the spleen has often been the focus of neuroimmune research because this secondary lymphoid organ is densely innervated by the sympathetic nervous system. In particular, at the electron microscopic level, it has been shown that noradrenergic nerve terminals form intimate contact with the surface membrane of T-lymphocytes and APCs of the peri-arteriolar lymphoid sheath of the spleen, with thickening of presynaptic cellular membranes and concentration of vesicles containing neurotransmitters at neuron–immune cell junctions (52, 76). This neuroimmune junction meets the criteria for synaptically-mediated neurotransmission, including local bidirectionality through cytokines and neurotransmitters from immune cells that modulate the release of sympathetic neurotransmitters from nerve terminals (53, 74, 76).

Cell–cell interactions *via* adhesion molecules are important in the maintenance of communication between cells (49, 78). Evidence already exists for direct cell adhesion between neurons and immune cells involving cell adhesion molecules [e.g., ICAM-1 (23, 54, 58, 79), ICAM-5 (67), VCAM (54, 58), selectins (66), and NCAM (23)]. Although little is known of the consequences of this interaction, it is, however, expected to be relevant in inflammation (54, 58) and neuronal damage (67, 79). The nervous system and immune system utilize these specialized cell surface contacts to directly convey and transduce highly controlled secretory signals between their constituent cell populations. The synaptic structure comprises central active zones of

exocytosis and endocytosis encircled by adhesion domains (80). Surface molecules that may be incorporated into and around the active zones contribute to modulation of the functional state of the synapse (49). The potential roles of adhesion molecules at synapses include stability, target recognition, and synaptic differentiation (81, 82). However, the mechanisms that localize molecules to specific subdomains remain unclear. Therefore, the identity of the cell adhesion molecules on the apposed membranes and their local concentration may be important determinants on synapse numbers and their location (81, 82).

The release of chemical mediators into the inflammatory milieu has been reported to increase the expression of various adhesion molecules (6, 26, 28). In particular, NCAM and ICAM may be important in mediating adequate analgesia in inflammatory pain by facilitating firm adhesion between opioid-containing immune cells and peripheral sensory neurons (23) (**Figure 2**). For example, intraplantar injection of the monoclonal antibody for NCAM, prior to the induction of inflammation, significantly reduced the antinociceptive response (paw pressure and paw thermal thresholds) produced by CRF or cold water swim stress in a dose-dependent manner (23). Anti-NCAM-treated rats responded normally to intraplantar fentanyl. In addition, β -END-containing immune cells within treated and untreated rats were histologically verified to have similar densities, suggesting no effect on leukocyte extravasation into inflamed tissue. *In vitro* studies showed a significant reduction in the number of lymphocytes adherence to DRG neuronal cultures following anti-NCAM and anti-ICAM-1 treatment compared to untreated cultures, thus supporting the notion that opioid-containing immune cells must adhere to peripheral sensory neurons to provide effective analgesia (23).

MODULATION OF IMMUNE CELL ADHERENCE BY OPIOIDS

Peripheral inflammatory pain can be effectively controlled by an interaction of opioids released by immune cells in close proximity to opioid receptors on peripheral sensory nerve terminals. Although direct contact between primary cultured DRG neurons and lymphocytes have been observed (23), whether this interaction is of functional relevance in peripheral inflammation is not yet established. This adhesion may also be partly mediated by opioid receptors, as shown by the effects of β -END on adhesion between cultured DRG neurons and lymphocytes (23). Exogenous application of β -END significantly attenuated lymphocyte adherence to nerve fibers compared to control, and this was completely and significantly reversed with naloxone. This may highlight an additional anti-inflammatory role for opioids in peripheral analgesia. Immune cell-derived opioids released locally may interfere with this direct neuroimmune interaction, resulting in dissociation and possibly migration of immune cells back to regional lymph nodes (1, 23). However, if direct cell adhesion itself does not elicit

opioid release from immune cells, then it is expected that agents such as CRF will trigger such release.

There is growing evidence that opioid peptides are potent modulators of cellular immune response, which can enhance or inhibit immune functions (83–89). Opioids including β -END (85, 86, 90, 91), met-enkephalin (85, 86, 91), and morphine (84) have been shown to modulate the adherence of immune cells to the endothelium. In particular, β -END and met-enkephalin, at physiological concentrations (10^{-8} and 10^{-6} M), enhanced the adherence and migration of human monocytes and neutrophils across capillary endothelial cells into inflamed tissues (85, 87). However, at higher concentrations of β -END (10^{-3} M) and met-enkephalin (10^{-5} M), chemotaxis of these immune cells into inflammatory sites decreased (85). The adherence of immune cell to the endothelium was suggested to involve opioid modulation of the expression of adhesion molecules, with quantitative studies confirming an increased number of integrin (CD11b and CD18) receptors on neutrophils at lower opioid concentrations (85). Furthermore, morphine has been demonstrated to attenuate leukocyte rolling and adhesion in both arterioles and venules *via* stimulation of nitric oxide production, which, in turn, down-regulates the expression of adhesion molecules (e.g., selectins and integrins) on endothelial cells (84). It is, therefore, likely that immune cell-derived opioids may attenuate the adherence of lymphocytes to DRG neurons following release within peripheral inflamed tissue (23).

CONCLUSION

Increasing evidence exists for a functional role in neuroimmune interactions between opioid-containing immune cells and peripheral sensory neurons within inflamed tissue. Since a fundamental goal is to understand synapse assembly at the molecular level, techniques such as electron microscopy, electrophysiology, and immunocytochemistry are powerful methods for characterizing structural, functional, and molecular attributes, respectively (92). This will provide novel insight into the peripheral mechanisms of immune-derived analgesia in inflammation, and the potential development of new therapeutic strategies utilizing this alternative analgesic pathway to counteract peripheral inflammatory pain.

AUTHOR CONTRIBUTIONS

SH was responsible for drafting and revising the article.

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Peripheral and Central Neuroinflammatory Changes and Pain Behaviors in an Animal Model of Multiple Sclerosis

Samuel S. Duffy, Chamini J. Perera, Preet G. S. Makker, Justin G. Lees, Pascal Carrive and Gila Moalem-Taylor*

School of Medical Sciences, UNSW Medicine, The University of New South Wales (UNSW Australia), Sydney, NSW, Australia

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Kevin A. Keay,
University of Sydney, Australia

*Correspondence:

Gila Moalem-Taylor
gila@unsw.edu.au

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Pain is a widespread and debilitating symptom of multiple sclerosis (MS), a chronic inflammatory demyelinating disease of the central nervous system. Although central neuroinflammation and demyelination have been implicated in MS-related pain, the contribution of peripheral and central mechanisms during different phases of the disease remains unclear. In this study, we used the animal model experimental autoimmune encephalomyelitis (EAE) to examine both stimulus-evoked and spontaneous pain behaviors, and neuroinflammatory changes, over the course of chronic disease. We found that mechanical allodynia of the hind paw preceded the onset of clinical EAE but was unmeasurable at clinical peak. This mechanical hypersensitivity coincided with increased microglial activation confined to the dorsal horn of the spinal cord. The development of facial mechanical allodynia also emerged in preclinical EAE, persisted at the clinical peak, and corresponded with pathology of the peripheral trigeminal afferent pathway. This included T cell infiltration, which arose prior to overt central lesion formation and specific damage to myelinated neurons during the clinical peak. Measurement of spontaneous pain using the mouse grimace scale, a facial expression-based coding system, showed increased facial grimacing in mice with EAE during clinical disease. This was associated with multiple peripheral and central neuroinflammatory changes including a decrease in myelinating oligodendrocytes, increased T cell infiltration, and macrophage/microglia and astrocyte activation. Overall, these findings suggest that different pathological mechanisms may underlie stimulus-evoked and spontaneous pain in EAE, and that these behaviors predominate in unique stages of the disease.

Keywords: multiple sclerosis, experimental autoimmune encephalomyelitis, pain, neuroinflammation, glia, T cells

INTRODUCTION

Multiple sclerosis (MS) is a chronic disease of the central nervous system (CNS) characterized by widespread focal areas of inflammation, demyelination, gliosis, and neurodegeneration. Typical onset is between the ages of 20 and 30, and it is the most common cause of chronic neurological disability in early to middle adult life (1–3). Of the multitude of sensory, cognitive, and motor symptoms associated with the disease, pain has recently been estimated to have a lifetime prevalence

of 66.5% (4). It severely impacts the quality of life of sufferers and is particularly difficult to treat (5, 6). Pain in MS may be nociceptive (arising as a result of non-neural tissue damage by activation of nociceptors), neuropathic (a consequence of direct damage to the somatosensory system), or mixed. Specific conditions include trigeminal neuralgia and Lhermitte's phenomenon (neuropathic; due to ectopic impulse generation along primary afferents), ongoing extremity pain (neuropathic; secondary to lesion formation in the spino-thalamo-cortical pathways), painful tonic spasms and spasticity pain (mixed; mediated by nociceptors and arises secondary to lesions in the central motor pathways), pain associated with optic neuritis (nociceptive; originating from *nervi nervorum*), musculoskeletal pains (nociceptive; secondary to motor disorders), and migraine (nociceptive; resulting from predisposition or secondary to midbrain lesions) (7). As it stands, there is a dire need for effective and targeted therapies aimed at the amelioration of pain in MS. This is an issue that, at least in part, stems from a lack of reliable and translatable pain outcome measures in animal models of MS.

Experimental autoimmune encephalomyelitis (EAE) is the most commonly used experimental animal model of MS, which shares many key pathological characteristics with the human disease; namely neuroinflammation, demyelination, and neuronal damage (8). Further, mice with EAE have been shown to develop pain behaviors including thermal hyperalgesia and mechanical and cold allodynia of the tail, hind paws, and fore paws in mild and preclinical EAE (9–14). An obvious limitation of these approaches arises from the confounding tail and hind limb motor impairment characteristic of clinical EAE, which impedes the testing of pain behaviors due to absent withdrawal reflexes during periods of paralysis. A recent study demonstrated that mice with EAE develop increased sensitivity to air puffs applied to the whisker pad indicating altered facial sensitivity in EAE (15). Although unclear, it is widely believed that stimulus-evoked pain behaviors in EAE occur as a result of inflammation, glial activation, and demyelination in areas involved in the processing of painful stimuli such as the dorsal horn of the spinal cord (SC) (10) and trigeminal afferent pathways (15). Most recently, the early emergence of neuropathic pain-like behaviors in the sequelae of EAE has been investigated and was shown to be associated with altered excitatory–inhibitory balance within the primary somatosensory cortex (16) and increased expression of the neuronal injury marker activating transcription factor-3 (ATF-3) in sensory neurons of the peripheral ganglia (17), both of which arose prior to an overt adaptive immune response and motor symptoms.

The study of pain in EAE has so far been focused on measures of hyperesthesia, which while nonetheless useful, may prove problematic when attempting to translate analgesic therapies optimized in mice to the treatment of spontaneous forms of neuropathic pain in MS patients. We hypothesized that mice with EAE develop spontaneous pain in addition to stimulus-evoked pain and aimed to examine such pain behaviors in association with neuroinflammatory changes over the course of chronic EAE. We show, for the first time, that in addition to stimulus-evoked pain (facial allodynia measured by whisker pad sensitivity to mechanical stimuli) mice with EAE also develop spontaneous

pain (facial grimacing measured by the mouse grimace scale, a facial expression-based pain coding system), an important component of MS-associated pain. Comprehensive analysis of immune, glial, and neural changes in correspondence with observable pain behaviors shows that distinct stages of EAE are associated with specific pain phenotypes and neuroinflammation in both the peripheral and CNS.

MATERIALS AND METHODS

Animals

Female C57BL/6J mice aged 10–12 weeks (Australian Biological Resources, Moss Vale, NSW, Australia) were used in all experiments. Mice were housed in individually ventilated cages with water and food *ad libitum* in groups of 3–5 and maintained on a 12-h light–dark cycle. The facility was kept at a constant room temperature (RT) and humidity, and the animals were monitored daily throughout experiments. All experiments were approved by the Animal Care and Ethics Committee of the University of New South Wales, Sydney, NSW, Australia.

EAE Induction and Assessment

Experimental autoimmune encephalomyelitis was induced by subcutaneous immunization with myelin oligodendrocyte glycoprotein 35–55 (MOG_{35–55}) emulsified in complete Freund's adjuvant (CFA). Emulsions were purchased from Hooke Laboratories (Lawrence, MA, USA) as prefilled syringes, each containing ~1 mg/mL MOG_{35–55} emulsified with 2–5 mg killed *Mycobacterium tuberculosis* H37Ra/mL in incomplete Freund's adjuvant. Control mice were immunized with CFA alone (Hooke Laboratories; at the same concentration given to mice immunized with MOG_{35–55}/CFA). Immunizations were given under 3–5% isoflurane anesthesia in oxygen as 2 × 100 µL subcutaneous injections; one at the base of the tail and one on the upper back. An intraperitoneal injection of 200 ng Pertussis Toxin (Hooke Laboratories) in 100 µL Dulbecco's Phosphate Buffered Saline (D-PBS; Life Technologies Pty Ltd., VIC, Australia) was given to all mice 2–6 h after subcutaneous immunization, and again 22–26 h later.

Post-induction, mice were monitored daily for body weight and EAE clinical scores according to a detailed EAE grading system supplied by Hooke Laboratories. Briefly, EAE clinical scores were assigned as follows: Grade 1 = limp tail; Grade 2 = limp tail and weakness of hind legs; Grade 3 = limp tail and complete paralysis of hind legs or limp tail with paralysis of one front and one hind leg; Grade 4 = limp tail and complete hind leg and partial front leg paralysis; Grade 5 = complete hind and complete front leg paralysis.

Measurement of Hind Paw Mechanical Allodynia

Hind paw mechanical allodynia was assessed by placing mice in 10 × 10 cm red-tinted chambers on an elevated wire mesh. Mice were habituated to the behavioral testing apparatus twice for 1 h each prior to baseline testing. Mechanical allodynia of the hind paws was assessed using a set of calibrated von Frey

filaments. These were applied to the mid-plantar surface of the hind paw until bending and maintained for 3 s. Paw withdrawals were noted as swift, sharp responses to application of the filament, and testing was conducted blind to experimental groups using Dixon's up-and-down method (18). A 50% paw withdrawal threshold (PWT) per animal was found by averaging the 50% PWT calculated for each paw.

Measurement of Facial Mechanical Allodynia

Facial allodynia was assessed using a method previously described by Lyons et al. to measure facial pain in a model of trigeminal inflammatory compression injury (19). In the week prior to baseline behavioral testing, animals were handled daily using a cotton glove in order to gradually acclimatize the mice to being gently restrained in the experimenter's hand. Prior to testing, the same experimenter gently restrained the mouse in their palm with the head exposed using the cotton glove until the mouse was acclimated and calm. During testing, a second experimenter blinded to experimental groups applied a 0.07 g von Frey filament to the whisker pad five times per side, with a 1-min interval between tests. Responses were recorded as head withdrawal, fore paw swiping, or facial flinching by a blinded experimenter, and a percentage of total responses over the five tests per side were calculated for each animal.

Mouse Grimace Scale

Mice were habituated for 15 min once prior to baseline testing in a 5 × 5 × 10 cm plastic arch with glass windows at each end placed on an elevated wire mesh. During testing, mice were placed in the same arch, with two Canon 500D cameras positioned at each end for high definition video recording. Mice were filmed for 11 min total, and screen grabs were later taken at each minute mark following beginning of recording for a total of 10 photos per mouse. These were taken as soon as a clear head shot could be observed and scored according to the criteria developed by Langford and colleagues (20) with the omission of the whisker change action unit as this was deemed difficult in C57BL/6J mice due to coat color (**Figure 2A**). Mean mouse grimace scale (MGS) difference scores from baseline were calculated in a blinded manner at time points tested post-EAE induction.

Flow Cytometry

At designated end-points post-EAE induction (day 8 pre-onset, day 16 clinical peak, and day 32 chronic phase), mice were euthanized using 0.1 mL of Lethobarb (Virbac, NSW, Australia), injected i.p. and were transcardially perfused with heparinized 0.9% saline solution. The L3–5 dorsal root ganglia (DRG; left and right), SC, brain, and trigeminal tissue (nerve and ganglia; right) were then dissected and placed into PBS on ice. Tissues were coarsely chopped and incubated with 1 mL of Accutase (Sigma Aldrich, NSW, Australia) for 30 min at 37°C and 5% CO₂. Tissues were then mechanically ground through 70 µm cell strainers (BD Biosciences, Franklin Lakes, NJ, USA) in 10 mL of PBS. Cell suspensions were centrifuged for 5 min at 1000 × g at 4°C, before discarding the supernatant. 10 mL of 30% Percoll

(GE Healthcare Australia Pty Ltd., NSW, Australia) in PBS was added to each sample, which were then centrifuged for 25 min at 600 × g at RT. The supernatant, including the myelin/cell debris layer, was carefully removed using a pipette, and the cell pellet was resuspended in 1 mL of autoMACS (magnetic-activated cell sorting; Miltenyi Biotec Australia Pty Ltd., NSW, Australia) running buffer. Samples were centrifuged for 5 min at 600 × g at 4°C, before removing as much of the supernatant from the pellet as possible using a pipette. Cells were counted and PBS added to give a final cell concentration of ~1 × 10⁷ cells per mL.

One hundred microliters of cells from each sample were divided into separate tubes, and 1 µL of Zombie Violet cell viability dye (Biolegend, CA, USA) was added to each tube, which were incubated in the dark for 15 min at RT. Cells were washed once in 1 mL of autoMACS running buffer by centrifuging for 5 min at 600 × g at 4°C, the supernatant was discarded and the residual volume was incubated with 1 µL anti-mouse CD16/CD32 Fc Block (eBioscience, CA, USA) for 5 min at RT. For analysis of premyelinating and myelinating oligodendrocytes, primary anti-galactocerebroside (GALC) and anti-MOG antibodies (Merck Millipore, VIC, Australia) were first conjugated to unique fluorophores using Lightning-Link Antibody Labeling kits (Novus Biologicals, Littleton, CO, USA) as per manufacturer's instructions. Anti-GALC-PE and anti-MOG-FITC conjugated antibodies were then incorporated into a standard flow cytometry staining protocol as follows. Antibodies including anti-CD45-APC (eBioscience; 1:1000), anti-CD4-FITC (eBioscience; 1:1000), Anti-GALC-PE (1:200), and anti-MOG-FITC (1:200) diluted in 100 µL of autoMACS running buffer were added to each sample, and cells were incubated for 30 min at 4°C in the dark. Appropriate isotype and fluorescence minus one controls were included by staining 100 µL of pooled samples for each tissue analyzed. After staining, cells were washed three times in 1 mL of autoMACS running buffer before being resuspended in 0.2 mL of the same buffer for analysis on a BD FACS Canto II flow cytometer. A minimum of 50,000 events was acquired per sample, and data were analyzed using FlowJo software (FlowJo, OR, USA).

Immunohistochemistry

At designated end-points post-EAE induction (day 8 pre-onset and day 16 clinical peak), mice were euthanized and transcardially perfused with heparinized 0.09% saline solution followed by 10% formalin solution (Sigma Aldrich). The L3–5 DRG, L3–5 SC, brain (including brain stem), and trigeminal tissue was then dissected and post-fixed in formalin solution overnight at 4°C. The medulla was then dissected from the brain, and all tissues were transferred to 30% sucrose + 0.1% sodium azide solution and stored at 4°C until sectioning. Sections were cut using a cryostat (Leica Biosystems, Buffalo Grove, IL, USA), with DRGs, and trigeminal tissue samples sectioned longitudinally at a thickness of 10 µm, and SC and medulla samples sectioned coronally at a thickness of 20 µm. All sections were cut sequentially such that each slide contained 4–6 serial sections representative of the entire thickness of the tissue. Sections were transferred directly to gelatin-coated glass slides, air dried overnight, and stored at –80°C until staining.

Prior to staining, sections were fixed with ethanol for 10 min at RT. Sections were then washed twice with distilled water and once with PBS containing 0.05% Tween-20 (PBS-T). A blocking solution containing PBS with 5% donkey or goat serum, 0.2% Tween-20, and 0.3% Triton X-100 was applied to each slide for incubation at RT for 1 h in the dark. The blocking solution was drained, and sections were incubated with the following antibodies diluted in PBS containing 5% bovine serum albumin and 0.03% Triton-X: rat anti-mouse CD3 (T cells; 1:100; R&D systems, MN, USA), rabbit anti-mouse/rat IBA-1 (ionized calcium-binding adaptor-1; macrophages/microglia; 1:2000; Wako Chemicals USA, Richmond, VA, USA), mouse anti-mouse GFAP (glial fibrillary acidic protein; activated astrocytes; 1:2000; Chemicon, Temecula, CA, USA), rabbit anti-mouse ATF-3 (activating transcription factor-3; cell damage; 1:400; Santa Cruz Biotechnology Inc., TX, USA), goat anti-mouse CGRP (calcitonin gene-related peptide; peptidergic neurons; 1:1000; Abcam, VIC, Australia), anti-mouse IBA-FITC (isolectin B4; non-peptidergic neurons; 1:100; Sigma Aldrich), and rabbit anti-mouse NF200 (neurofilament 200; myelinated neurons; 1:1000; Sigma Aldrich). IBA-1 and GFAP primary antibody incubation was conducted at RT for 1 h, while all other primary antibodies were incubated overnight at 4°C. Sections were then washed four times with PBS-T for 10 min each, before adding secondary antibodies, which included: Alexa Fluor 488 conjugated donkey anti-mouse (1:1000; Life Technologies, Mulgrave, VIC, Australia), Alexa Fluor 546 conjugated donkey anti-rabbit (1:1000; Life Technologies), or Cy3 conjugated donkey anti-rabbit (1:1000; Jackson ImmunoResearch Laboratories Inc., PA, USA) in the same buffer as the primary antibody. Negative staining controls were incorporated whereby tissues were incubated with secondary antibody alone in the absence of the relevant primary antibody (to control for non-specific binding of the secondary antibody). Following a 1 h incubation at RT in the dark, sections were washed four times in PBS-T for 10 min each. Prolong gold anti-fade reagent with 4', 6-diamidino-2-phenylindole (DAPI; Life Technologies) was applied before slides were cover slipped and stored at 4°C until viewing.

Image Analysis

Analysis of images was conducted blind to experimental groups from which the tissue was derived.

Trigeminal Ganglia and Dorsal Root Ganglia

For each animal, slides containing 4–6 sections of the L3–5 DRGs (left and right, with two of each L3–5 DRG represented in each section) or trigeminal tissue (left) were stained for a given antibody. For DRGs, images were taken of 2–3 DRGs contained in each section (a total of 12–18 images per slide/animal). In trigeminal ganglia (TG), images of areas containing cell bodies (inclusive of the ophthalmic, maxillary, and mandibular distributions of ganglia) were taken at 20× magnification for each section (a total of 12–18 images per slide/animal). Using Image J software, immunoreactive cells were either manually counted and expressed per area of ganglion (CD3 analyses), counted and expressed as a percentage of total cell bodies in the ganglia of interest (ATF-3 and neuropeptide analyses), or expressed as a percentage of immunoreactivity after subtracting background

(IBA-1 analyses). Values for each image of ganglia regions were averaged to give a mean immunoreactivity per animal.

Trigeminal Nerve and Trigeminal Root Entry Zone

Slides containing 4–6 sections of left trigeminal tissue per animal were stained for a given antibody. Images were taken at 20× magnification of regions immediately distal to the CNS-peripheral nervous system (PNS) junction (trigeminal nerve; TN images) and just proximal to the CNS–PNS junction at the trigeminal root entry zone (TREQ) (4–6 images per slide/animal). Immunoreactive cells were either manually counted and expressed per area of nerve (CD3 analyses) or expressed as a percentage of immunoreactivity after subtracting background (IBA-1 analyses) using Image J software. Values for each section were averaged to give the mean immunoreactivity per animal.

Spinal Cord and Spinal Trigeminal Nucleus

Slides containing 4–6 sections of L3–5 SC or medulla per animal were stained for a given antibody. For SC sections, images were taken at 20× magnification of the left and right ventral and dorsal horn regions (8–12 images of ventral horn, and 8–12 images of dorsal horn regions per animal/slide). Sections of the medulla were taken around the level of the obex in the caudal medulla in order to visualize the region corresponding to the spinal trigeminal nucleus (STN) (sections were inclusive of the subnucleus caudalis, subnucleus interpolaris, and subnucleus oralis). Images were taken at 20× magnification in regions of the left and right STN (8–12 images per animal/slide). Image J software was used to calculate the percentage of total immunoreactivity after subtracting background. Values for each region were averaged across sections to give the mean immunoreactivity per animal.

Image Acquisition

All images were viewed using Olympus BX51 epifluorescence microscope, and images captured using an Olympus DP73 camera using cellSens software (Olympus, Tokyo, Japan). All images for a particular stain were taken using identical microscope settings.

Statistical Analysis

All statistical analyses were conducted using GraphPad Prism 6 software. Since pain behavioral data sets involving the testing of mechanical allodynia proved to be not normally distributed using a Shapiro–Wilk normality test, a non-parametric Mann–Whitney test was used as the most appropriate statistical test for comparing two groups – EAE mice vs. control mice at each time point. The Holm–Bonferroni correction for multiple comparisons was then used to account for multiple testing. MGS data were analyzed using a repeated measures two-way ANOVA (treatment; time) with Tukey's *post hoc* test. Immunohistochemistry data were analyzed using an unpaired student's *t*-test (when comparing two groups; EAE mice vs. control mice) or a one-way ANOVA with Tukey's *post hoc* test (when comparing greater than two groups in neuropeptide analyses) at individual time points. Multiple time point flow cytometry data were analyzed using two-way ANOVA (treatment; time) with Tukey's *post hoc* test. Pain behavioral data sets analyzed using non-parametric statistical tests (hind paw and facial allodynia) are presented as box and whisker plots, where

box limits show the first and third quartile, the center line is the median and the whiskers represent the minimum and maximum values. MGS, flow cytometry, and immunohistochemistry data are presented as arithmetic mean \pm SEM. The level of significance was set as $p < 0.05$ for all analyses.

RESULTS

Increased Stimulus-Evoked Pain and Facial Grimacing in Mice with EAE

To investigate pain symptoms during the course of chronic EAE, mice were immunized with the MOG_{35–55} peptide in CFA. Mice developed chronic EAE with typical disease onset between 10 and

13 days post-induction, a mean peak clinical score of 3 on day 16, and a gradual, partial recovery to a mean clinical score of 2 by day 32 post-induction. Control mice immunized with CFA alone did not develop any clinical signs of EAE over the 32-day monitoring period (**Figure 1A**). Clinical EAE was accompanied by a significant loss of body weight, which was most severe at time points corresponding to the EAE clinical peak (**Figure 1B**).

To assess stimulus-evoked pain, we first tested mechanical pain hypersensitivity in the hind paws (**Figure 1C**). Compared to controls, EAE mice showed reduced 50% PWTs during the pre-clinical stage of disease on days 8 and 10 post-induction ($n = 6–15$, $p < 0.01$). Corresponding to the onset of clinical EAE and accompanying hind limb motor impairment, the 50% PWTs of EAE mice showed a sharp rise on day 15 (clinical peak

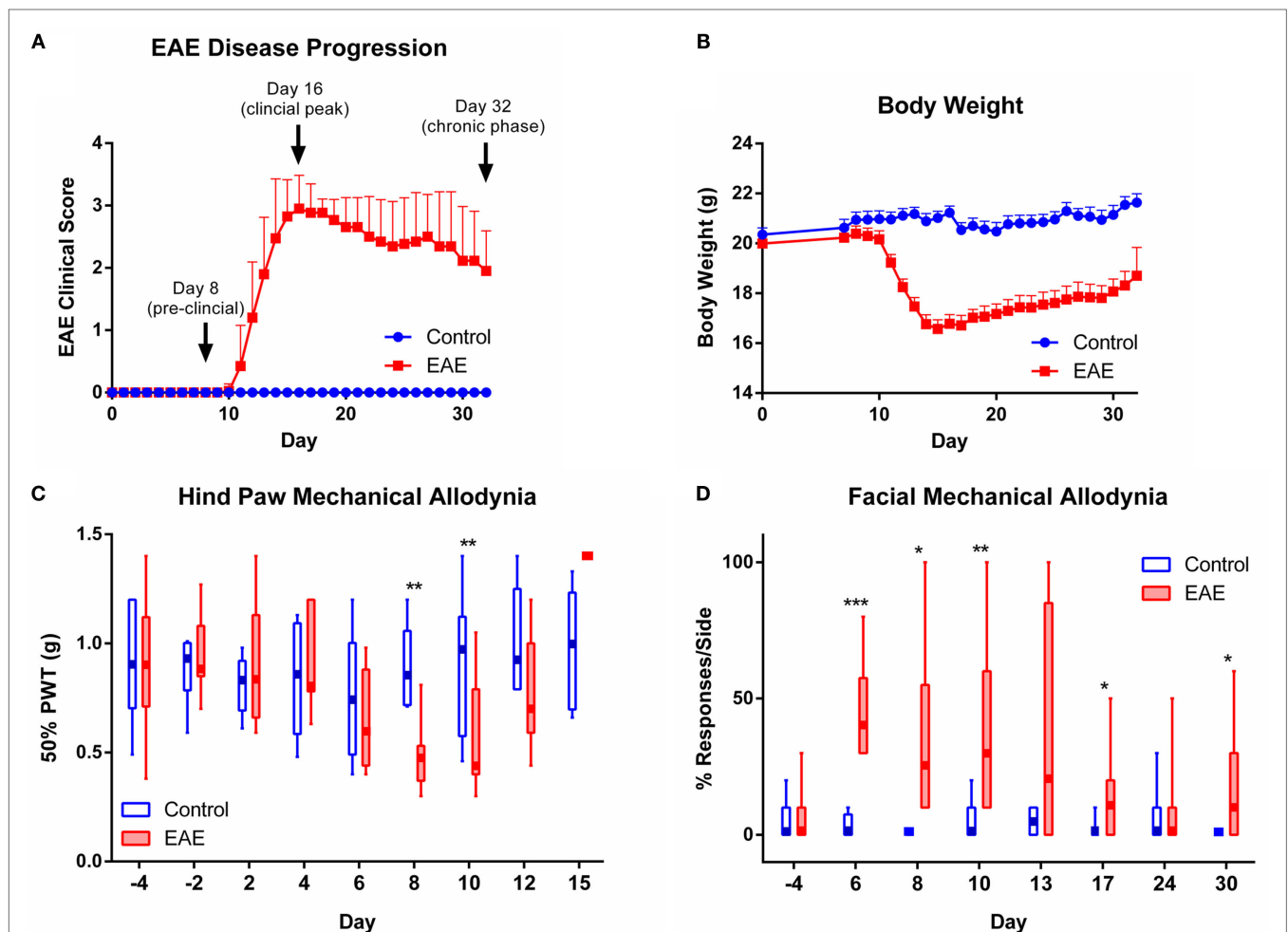


FIGURE 1 | EAE disease progression and evoked pain behaviors observed over the course of chronic EAE. Disease progression of EAE (**A**) and (**B**) body weight over the 32-day monitoring period post-induction in control and EAE mice ($n = 20$). Time points chosen for analysis of nervous tissue at preclinical, peak, and chronic phases of disease are indicated in (**A**). (**C**) Mechanical allodynia of the hind paw observed in the preclinical period in mice with EAE. At days 8 and 10 post-induction, EAE mice showed significantly reduced 50% paw withdrawal thresholds (PWTs) compared to control mice ($n = 6–15$). (**D**) Facial allodynia observed in mice over the course of chronic EAE. At days 6, 8, 10, 17 and 30 post-induction, EAE mice recorded a significantly increased percentage of responses to the mechanical stimulus applied to the whisker pad compared to control mice ($n = 4–9$). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, Mann-Whitney test with Holm-Bonferroni correction for multiple comparisons. EAE clinical scores and body weight data are expressed as mean \pm SEM, while pain behavioral data are expressed as box and whisker plots where box limits show the first and third quartile, the center line is the median, and the whiskers represent the minimum and maximum values.

of disease) where PWTs rose above baseline levels and reached the imposed upper limit of von Frey testing in all animals (1.4 g; **Figure 1C**). Testing of mechanical pain hypersensitivity in the hind paws was terminated at disease peak due to paralysis. The restrictions imposed by hind limb motor confounds on continual testing of mechanical allodynia in clinical EAE prompted us to use a technique developed for testing mechanical pain in the face (19), which is not affected by motor deficits. We found that EAE mice were significantly more responsive to mechanical stimuli applied to the whisker pad than control mice over the entire course of chronic EAE, as seen on days 6, 8, 10, 17, and 30 (**Figure 1D**, $n = 4-9$, $p < 0.05-0.001$).

To assess spontaneous (as opposed to experimenter-evoked) pain in EAE, we utilized the MGS (**Figure 2A**), a standardized murine facial expression-based coding system (20). This allowed both continuous testing of pain behaviors during paralytic clinical

disease, and the assessment of spontaneous pain in EAE, which has not yet been explored in the model. No change in mean MGS scores were seen in the preclinical period (day 8); however, the mean MGS scores in EAE mice were higher at day 16 ($n = 7-8$, $p < 0.01$) and day 30 ($n = 7-8$, $p < 0.01$) post-EAE induction as compared to control mice (**Figure 2B**).

Changes in the Proportions of Premyelinating and Myelinating Oligodendrocytes in the CNS of Mice with EAE

Given the fact that oligodendrocyte damage in the absence of an innate or adaptive immune response is sufficient to confer central pain behaviors in mice (21), we next investigated whether oligodendrocyte changes are associated with the pain

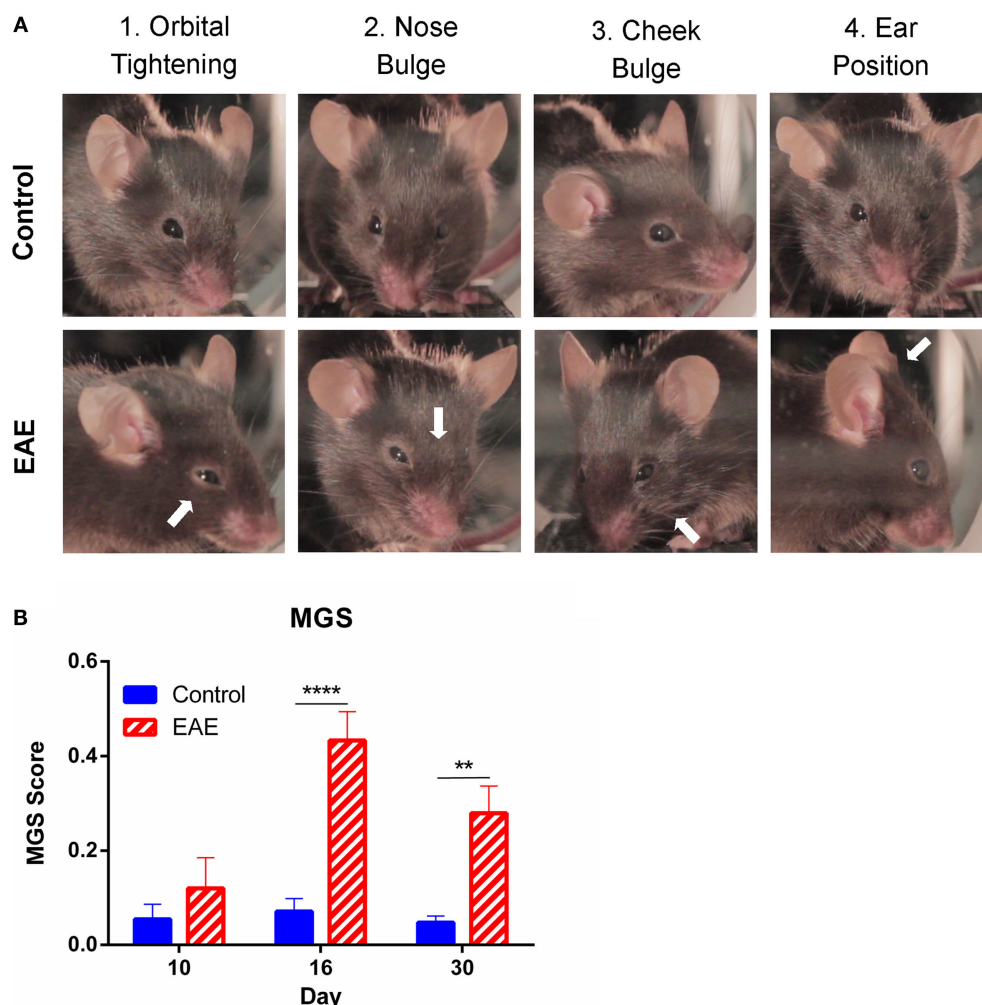


FIGURE 2 | Facial grimacing observed over the course of chronic EAE. (A) Representative images of action units analyzed as part of the mouse grimace scale (MGS) scoring. Control images depict an MGS score of 0 for each of the given action units, while white arrows indicate action units which scored 1–2 on the MGS in EAE mice. **(B)** Mean MGS difference scores calculated from control and EAE mice over the course of chronic EAE. Significant increases in MGS scores were seen at days 16 and 30 in EAE mice compared to control mice ($n = 7-8$). ** $P < 0.01$ and **** $P < 0.0001$, repeated measures two-way ANOVA followed by Tukey's *post hoc* test. Data are expressed as mean \pm SEM.

behaviors observed in our chronic EAE model. Flow cytometry was used to quantify changes in CNS myelination, as previously described (22). $CD45^{low}GALC + MOG^{low}$ premyelinating and $CD45^{low}GALC + MOG^{high}$ myelinating oligodendrocyte populations in the brain and SC were assessed over the course of chronic EAE. Mononuclear cells were first gated, followed by selection of live singlets (Figures 3A–C). $CD45^{low}$ resident CNS cells positive

for GALC were then gated (Figure 3D) and analyzed for changes in MOG positivity between control (Figure 3E) and EAE mice (Figure 3F).

No changes in myelination were found in the brain or SC at the preclinical stage of EAE (day 8 post-EAE induction, $n = 5$). At the clinical peak (day 16 post-induction), decreases in $GALC + MOG^{high}$ oligodendrocytes were seen, accompanied

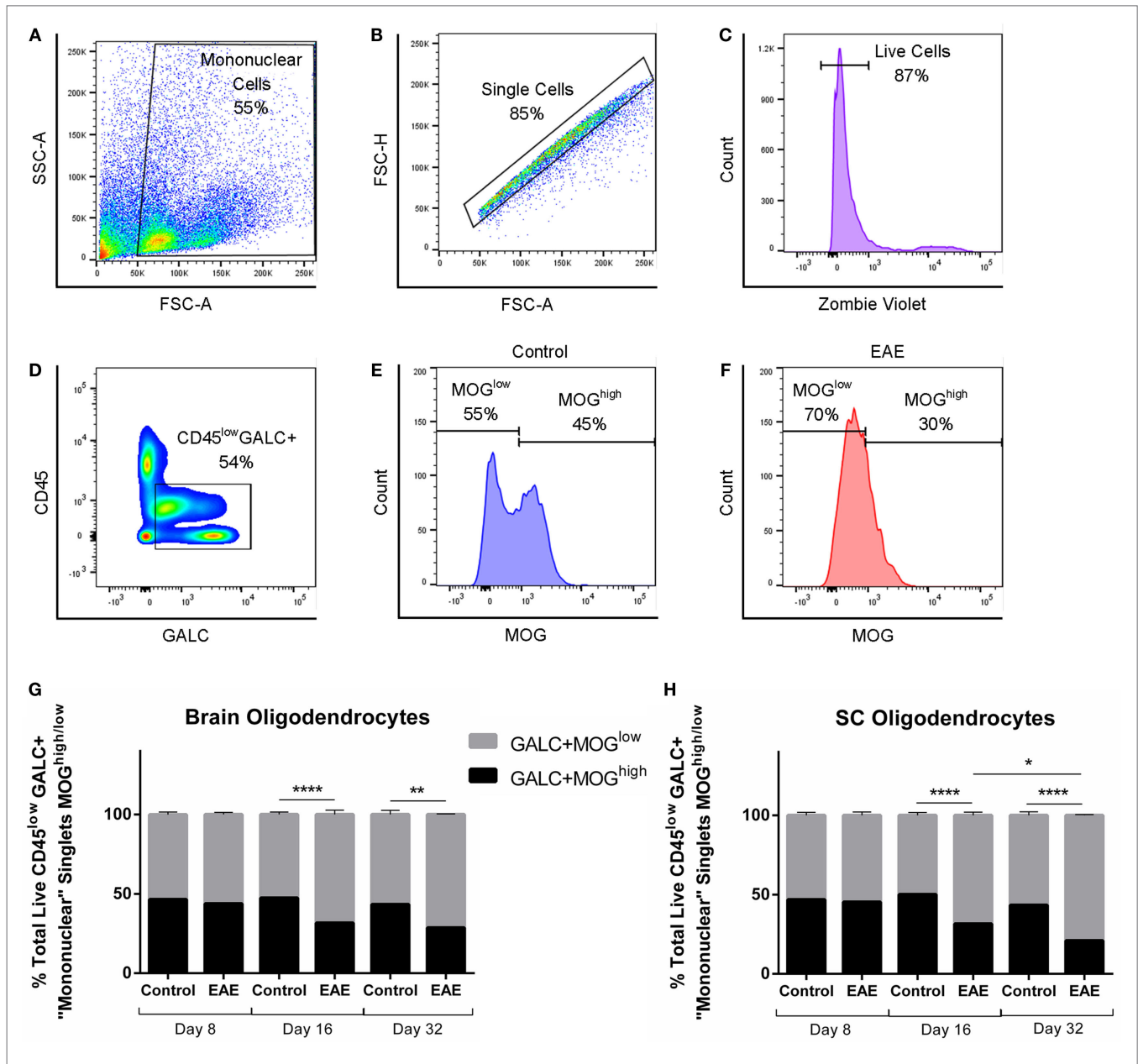


FIGURE 3 | The proportion of premyelinating to myelinating oligodendrocytes over the course of chronic EAE. Flow cytometry to identify proportions of $GALC + MOG^{low}$ premyelinating to $GALC + MOG^{high}$ myelinating oligodendrocytes in the brain and spinal cord (SC) was carried out in EAE and control mice. Mononuclear cells were first gated (A), followed by consecutive gating of singlets (B), live cells (C), and $CD45^{low}GALC +$ cells for further analysis (D). Representative histograms showing numbers of MOG^{high} cells and MOG^{low} cells in control mice (E) and EAE mice (F). Bar graphs showing significant decreases in MOG^{high} myelinating, and subsequent significant increases in MOG^{low} premyelinating, oligodendrocytes in the brain (G), and SC (H) of EAE mice compared to control mice at day 16 and 32 post-induction. No difference was seen between experimental groups preclinically at day 8. In the SC (H), a significant decrease in MOG^{high} myelinating and subsequent significant increase in MOG^{low} premyelinating oligodendrocytes was also observed in EAE mice at day 32 compared to EAE mice at day 16. * $P < 0.05$, ** $P < 0.01$, and **** $P < 0.0001$, two-way ANOVA followed by Tukey's *post hoc* test, $n = 3-5$. Data are expressed as mean \pm SEM.

by a subsequent increase ($n = 5$, $p < 0.0001$) in the proportion of GALC+ MOG^{low} premyelinating oligodendrocytes in the brain (Figure 3G) and SC (Figure 3H). This was also apparent in the chronic phase of EAE (day 32 post-induction) in the brain (Figure 3G, $n = 3$, $p < 0.01$) and SC (Figure 3H, $n = 3$, $p < 0.0001$). Interestingly, despite the partial recovery typical of mice entering the chronic phase of EAE, this regain of hind limb movement was associated with progressive and continued loss of GALC+ MOG^{high} myelinating oligodendrocytes in the SC (Figure 3H, $n = 3$, $p < 0.05$).

The lack of a difference in oligodendrocyte subtypes in the pre-clinical stage suggests that CNS demyelination has not yet taken place at day 8 and does not correspond with the early emergence of allodynia in the sequelae of our EAE model.

Peripheral and Central Changes in CD4+ Cell Infiltration in Mice with EAE

To investigate changes in neuroinflammation, we first assessed infiltration of cells expressing CD4 (a marker expressed predominantly on the surface of helper T cells as well as minor populations of other immune cells) into the CNS and peripheral ganglia over the course of chronic EAE using flow cytometry. Mononuclear cells were gated (as in Figure 3A) and single cells selected (as in Figure 3B), before gating viable cells (as in Figure 3C) for inclusion in further analysis for CD4 positivity in control and EAE mice (Figures 4A,B, respectively). Results showed that CD4+ cell infiltration was not apparent in the preclinical period, yet was clearly seen at the EAE clinical peak in all tissues analyzed including the brain, SC, trigeminal tissue, and DRG (Figures 4C–F, $n = 4–6$). CD4+ cells were also increased in the brain and SC in the chronic phase of the disease in EAE mice compared to control animals (Figures 4C,D, $n = 6$). A trend for increased CD4+ cells was seen in the trigeminal tissue of EAE mice compared to control animals in the chronic phase of disease, although this failed to reach significance (Figure 4E, $n = 6$, $p = 0.0559$). In the brain and DRG, CD4+ cells were reduced in the chronic phase compared to the EAE clinical peak (Figures 4C,F, $n = 5–6$). The absence of significant CD4+ cell infiltration at day 8 suggests that the preclinical allodynia observed in EAE mice is unlikely to arise from a central adaptive immune response.

Peripheral and Central Changes in Macrophage and Glial Activation and T Cell Infiltration in Preclinical EAE

To determine whether an early response mediated by immune activation is associated with the preclinical allodynia observed in the hind paw and whisker pad, immunostaining for IBA-1 (macrophage/microglia marker) and GFAP (astrocyte marker) was carried out. Areas analyzed for IBA-1 included the dorsal (DHSC) and ventral (VHSC) horns of the L3–5 SC (Figure 5A), STN (Figure 5B), TG, TN, and TREZ (Figure 5C). There was no difference between EAE and control mice in IBA-1+ immunoreactivity in any of the tissue regions analyzed, except for an increase in IBA-1 expressing cells in the dorsal horn of the SC of EAE mice compared to control mice (Figures 5D,E,H, $n = 4–5$, $p < 0.05$). Analysis of GFAP expression in the dorsal and ventral

horns of the SC revealed no differences in astrocyte activation between EAE and control mice (Figure 5I, $n = 4–5$).

Since flow cytometric analysis of the CNS and peripheral ganglia showed no increase in CD4+ cells at day 8 (Figure 4), immunostaining for CD3+ T cells was carried out with a focus on specific regions of the trigeminal afferent pathway (Figure 5C). This was done to ascertain whether the preclinical facial allodynia of EAE mice may be linked to a subtle early T cell infiltration into the peripheral trigeminal afferent pathway occurring prior to more overt central infiltration. A small but significant increase in CD3+ cells was found restricted to the TN (Figures 5F,G,J, $n = 4–5$, $p < 0.01$), and a similar trend, though not statistically significant, was seen in the TG (Figure 5J, $n = 4–5$).

Peripheral and Central Changes in Macrophage and Glial Activation and T Cell Infiltration in Clinical EAE

Immunohistochemical analysis to characterize changes in activation of macrophages/microglia in the SC and trigeminal afferent pathway and astrocytes in the SC at the clinical peak of EAE was also conducted. A substantial increase ($n = 4–5$, $p < 0.0001$) in IBA-1 expressing cells in both the ventral (Figures 6A,B,Q) and dorsal horns (Figures 6C,D,Q) of the SC in EAE mice compared to control mice was found. An increase in IBA-1 immunoreactivity was also apparent in the TN (Figures 6E,F,Q, $n = 4–5$, $p < 0.05$) and TREZ (Figures 6G,H,Q, $n = 4–5$, $p < 0.05$). GFAP+ cells were similarly increased in the ventral (Figures 6I,J,R, $n = 4–5$, $p < 0.001$) and dorsal (Figures 6K,L,R, $n = 4–5$, $p < 0.001$) horns of the SC in EAE mice compared to control mice.

Since T cell infiltration into the CNS at the EAE clinical peak has been demonstrated by numerous studies and by our flow cytometric analysis (Figure 4), we carried out immunohistochemistry for CD3 in the trigeminal afferent pathway and L3–5 DRGs. We found CD3+ cells in increased numbers within the TG (Figures 6M,N,S, $n = 4–5$, $p < 0.05$) and TREZ (Figures 6O,P,S, $n = 4–5$, $p < 0.001$) in EAE mice compared to control mice. A trend for increased CD3+ cells in the TN was also found, although this failed to reach significance (Figure 6S, $n = 4–5$). Immunostaining for CD3+ cells in the DRG showed no significant difference in T cell numbers between EAE and control mice (Figure 6S, $n = 4–5$).

As significant changes in T cell infiltration were observed in the TG in clinical EAE (day 16), we next examined whether ATF-3 (a marker of neurons with damaged primary afferents) is induced in peptidergic (CGRP+) and non-peptidergic (IB4+) small diameter neurons, and NF200+ large diameter myelinated neurons using immunohistochemistry. This was of interest as injured DRG neurons have been linked to pain behaviors in mice with peripheral nerve injury (23) and rats with brachial plexus avulsion (24). We found a small, though significant, increase in the percentage of total ATF-3 immunopositive cell bodies in the TG of EAE ($1\% \pm 0.2$) compared to control ($0.1\% \pm 0.05$) mice (Unpaired Student's *t*-test, $n = 4–5$, $p < 0.01$). Further analysis revealed ATF3+ neurons present in EAE mice (Figure 7B), but not controls (Figure 7A), predominantly co-expressed NF200. An increase in NF200+ATF-3+ cell bodies compared

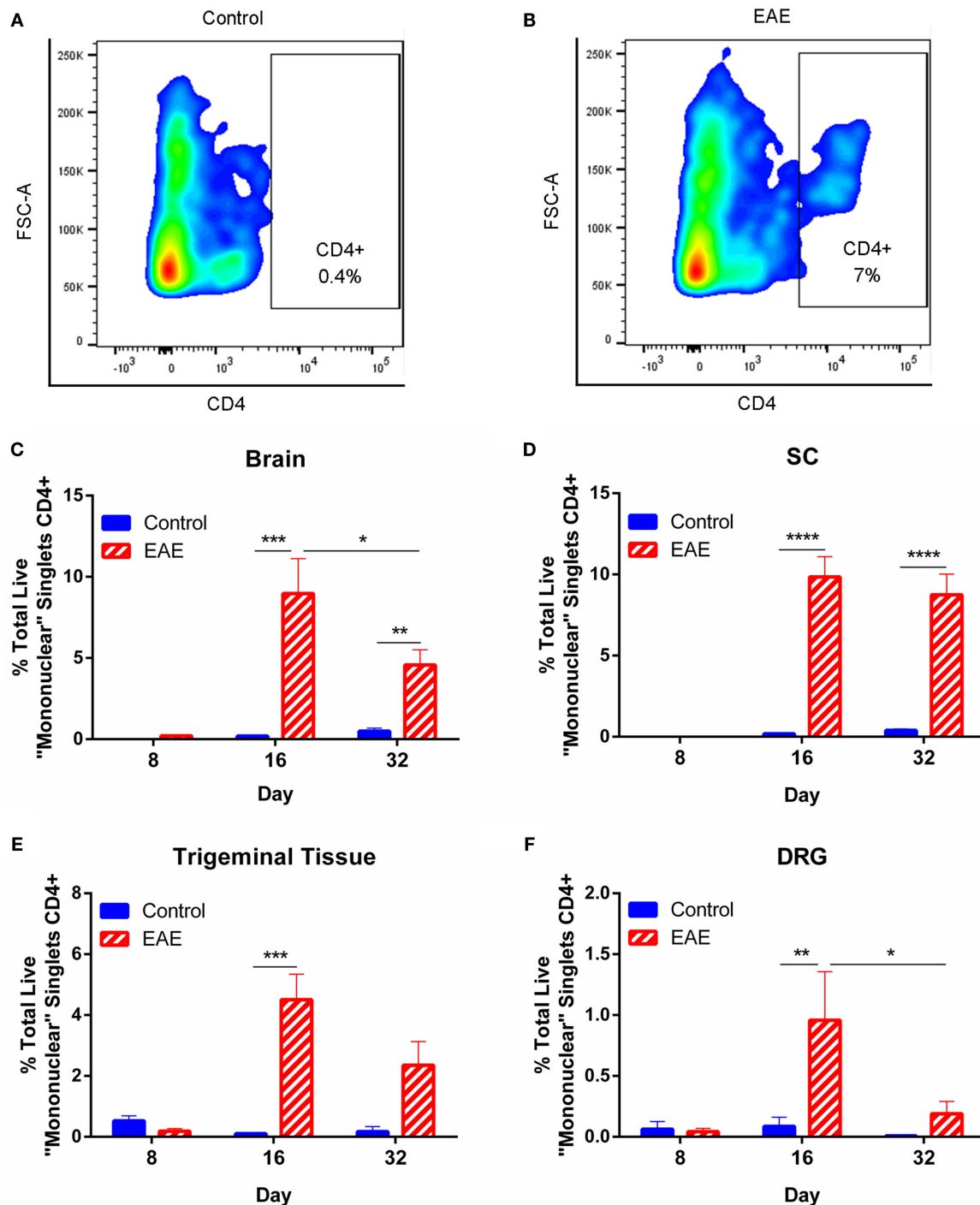


FIGURE 4 | CD4+ immune cell infiltration over the course of chronic EAE. Flow cytometry to identify infiltrating CD4+ cells in the brain, spinal cord (SC), trigeminal tissue, and dorsal root ganglia (DRG) was carried out in EAE and control mice. Mononuclear cells were first gated, followed by singlets and live cells (as in **Figures 3A–C**). Representative contour plots from trigeminal tissue showing an absence of CD4+ cells in control animals (**A**) and the presence of CD4+ cells in EAE mice (**B**). The percentage of CD4+ cells was significantly increased in the brain (**C**) and SC (**D**) of EAE mice compared to control mice at days 16 and 32 post-induction. The percentage of CD4+ cells was significantly reduced in the brain (**C**) of EAE mice at day 32 compared to EAE mice at day 16. The percentage of CD4+ cells was significantly increased in the trigeminal tissue (**E**) and in the DRG (**F**) of EAE mice compared to control mice at day 16 post-induction. In the DRG (**F**), the percentage of CD4+ cells was significantly reduced in EAE mice at day 32 post-induction compared to EAE mice at day 16 post-induction. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$, two-way ANOVA followed by Tukey's *post hoc* test, $n = 4–6$. Data are expressed as mean \pm SEM.

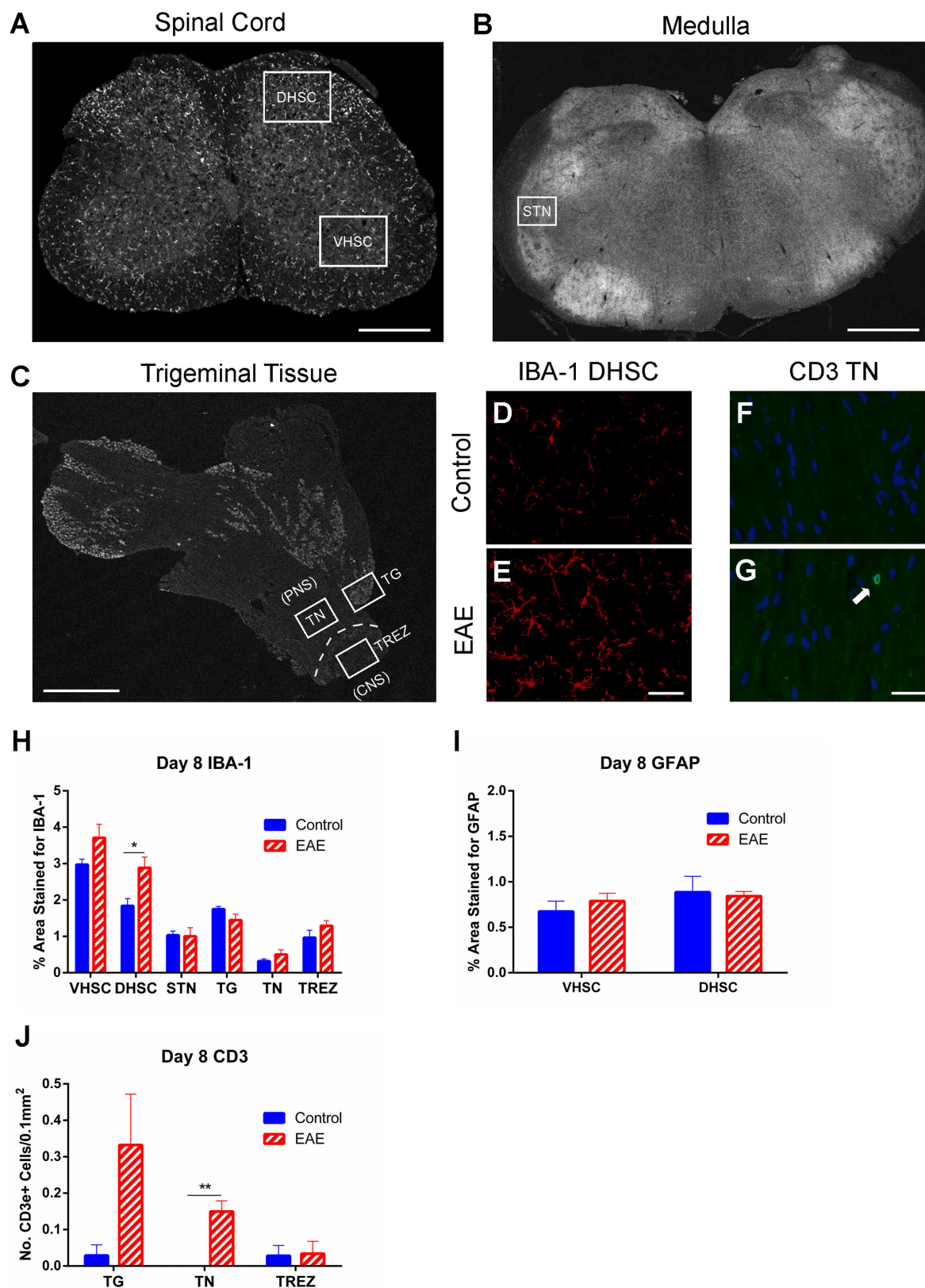


FIGURE 5 | Macrophage and glial activation and T cell infiltration in preclinical EAE.

(Continued)

FIGURE 5 | Continued

IBA-1 and GFAP immunohistochemistry in the L3–5 spinal cord (SC), and IBA-1 and CD3 immunohistochemistry in the trigeminal afferent pathway were carried out on day 8 (preclinical EAE) in EAE and control mice. **(A)** Representative image of a section of L3–5 SC depicting areas of the ventral horn (VHSC) and dorsal horn (DHSC) selected for analysis. Scale bar equals 500 μ m. **(B)** Representative image of a section of medulla showing the region corresponding to the spinal trigeminal nucleus (STN). Scale bar equals 1 mm. **(C)** Representative image depicting the peripheral trigeminal afferent pathway, inclusive of the trigeminal ganglia (TG), proximal trigeminal nerve (TN), and trigeminal root entry zone (TREZ). Scale bar equals 1 mm. Representative image of the DHSC taken from a control mouse **(D)** showing observably less IBA-1 staining than an EAE mouse **(E)**. Scale bar equals 50 μ m. Representative image of a region of TN taken from a control mouse **(F)** showing an absence of CD3+ cells, which were present in EAE mice **(G)**. Scale bar equals 25 μ m and white arrow indicates a CD3 immunoreactive cell with DAPI nuclear staining. **(H)** Bar graph summarizing levels of IBA-1 immunostaining, which were significantly increased in the DHSC of EAE mice; however, no difference was seen in any of the other tissues analyzed. **(I)** Bar graph depicting levels of GFAP immunostaining in the VHSC and DHSC, where no significant difference was seen between control and EAE mice. **(J)** Bar graph showing a significant increase in CD3+ cells in the TN, and a trend for increased numbers of CD3+ cells in the TG ($p = 0.08$). No difference was seen between experimental groups in the TREZ. * $P < 0.05$ and ** $P < 0.01$, unpaired student's t -test, $n = 4$ –5. Data are expressed as mean \pm SEM.

to CGRP+ATF-3+ ($n = 4$ –5, $p < 0.0001$), and IB4+ATF-3+ ($n = 4$ –5, $p < 0.01$) cell bodies was seen in the TG of mice at the clinical peak of EAE (**Figure 7C**) suggesting that neuronal damage is selective for myelinated NF200+ neurons.

DISCUSSION

Although it is well established that neuroinflammation and demyelination are important contributors to pain behaviors in MS and EAE (25, 26), the relationship between the pain phenotypes at different stages of disease and potential peripheral and central contributors is unclear. We found that stimulus-evoked and spontaneous pain in EAE may derive from unique pathological mechanisms, with the former arising in the absence of overt central lesion formation. A subtle increase in IBA-1+ cell numbers was seen in the dorsal horn of the SC in preclinical disease, and this corresponded to increased allodynia of the hind paw. Analysis of the trigeminal afferent pathway revealed that facial allodynia coincided with peripheral changes including a small, but significant, infiltration of T cells into the TN during preclinical disease and specific damage to myelinated NF200+ neurons at the EAE clinical peak. Clinical EAE and the onset of spontaneous pain appear to be associated with more overt changes, with potential peripheral and central contributions.

This study was conducted in female mice to account for the fact that MS is universally more prevalent in women than men (27). We show here that female mice display both stimulus-evoked and non-evoked pain behaviors throughout the course of chronic EAE, and that facial allodynia and facial grimacing predominate in unique stages of the disease. This finding suggests different underlying etiologies for EAE-induced evoked pain and spontaneous pain. Similarly to Thorburn and colleagues (15), we describe facial allodynia and trigeminal pathology in mice with EAE as described in patients suffering from trigeminal neuralgia associated with MS (28–31). In addition to effectively modeling trigeminal neuralgia, the use of von Frey filaments to elicit facial allodynia allows continual testing of cutaneous hypersensitivity during periods of otherwise confounding lower body motor impairment. Interestingly, the development of facial and hind paw allodynia arose prior to the onset of clinical symptoms, in line with a previous study that showed both mechanical and cold allodynia of the hind paw in the preclinical period of EAE (10). We also present the utility of the MGS (20) in measuring spontaneous pain over the course of

chronic EAE, which may increase the translational capacity of pain research in EAE when testing analgesic therapies for MS. Facial palsy occurs in some patients with MS (32) and may be due to peripheral lesions affecting motor facial nerve fibers or central lesions in the area of the facial nucleus (33). While EAE is typically considered a central demyelinating disorder, we cannot exclude the possibility that increased facial grimacing may be influenced by demyelination of lower motor neurons resulting in brainstem reflex abnormalities and a compromised ability of EAE mice to exhibit facial expressions. Regardless, such demyelination is also known to be a major contributor to neuropathic pain (34), and therefore does not discount the interpretation of increased facial grimacing as a response to pain. Additionally, it remains unclear whether the facial grimacing observed in EAE mice is reflective of spontaneous pain similar to trigeminal neuralgia or of spontaneous pain arising in area(s) unrelated to the face. As the MGS was reported to be non-useful for measuring neuropathic pain in peripheral nerve injury models (20), we cannot discount that the facial grimacing induced in EAE mice may be due to nociceptive pain arising as a consequence of musculoskeletal problems secondary to lower body paralysis and spasticity. Given that the majority of MS patients with central pain also suffer concurrent paresis (35), it is perhaps desirable to measure pain in stages of EAE where lower body motor impairment is apparent as has been made possible using the methods described in the current study.

A recent study demonstrated that conditional ablation of oligodendrocytes leads to the development of pain behaviors in mice. This suggests that a loss of oligodendrocyte function, which maintains axonal integrity in the CNS, is able to trigger the development of neuropathic pain (21). We therefore assessed demyelination over the course of chronic EAE using flow cytometry. Although we found decreases in GALC+ MOG^{high} myelinating oligodendrocytes in the brain and SC in the peak and chronic phases of clinical EAE, no changes were observed in the preclinical stage. Accompanying these clinical decreases in myelination was a subsequent increase in GALC + MOG^{low} premyelinating oligodendrocytes. This suggests an arrest of oligodendrocyte maturation at the premyelinating stage, presumably due to the presence of autoreactive T cells sensitized to the MOG antigen. Since overt demyelination was only observed in periods of clinical EAE, it appears that allodynia, which predominates during the preclinical phase of the disease, is not directly related to oligodendrocyte dysfunction or damage.

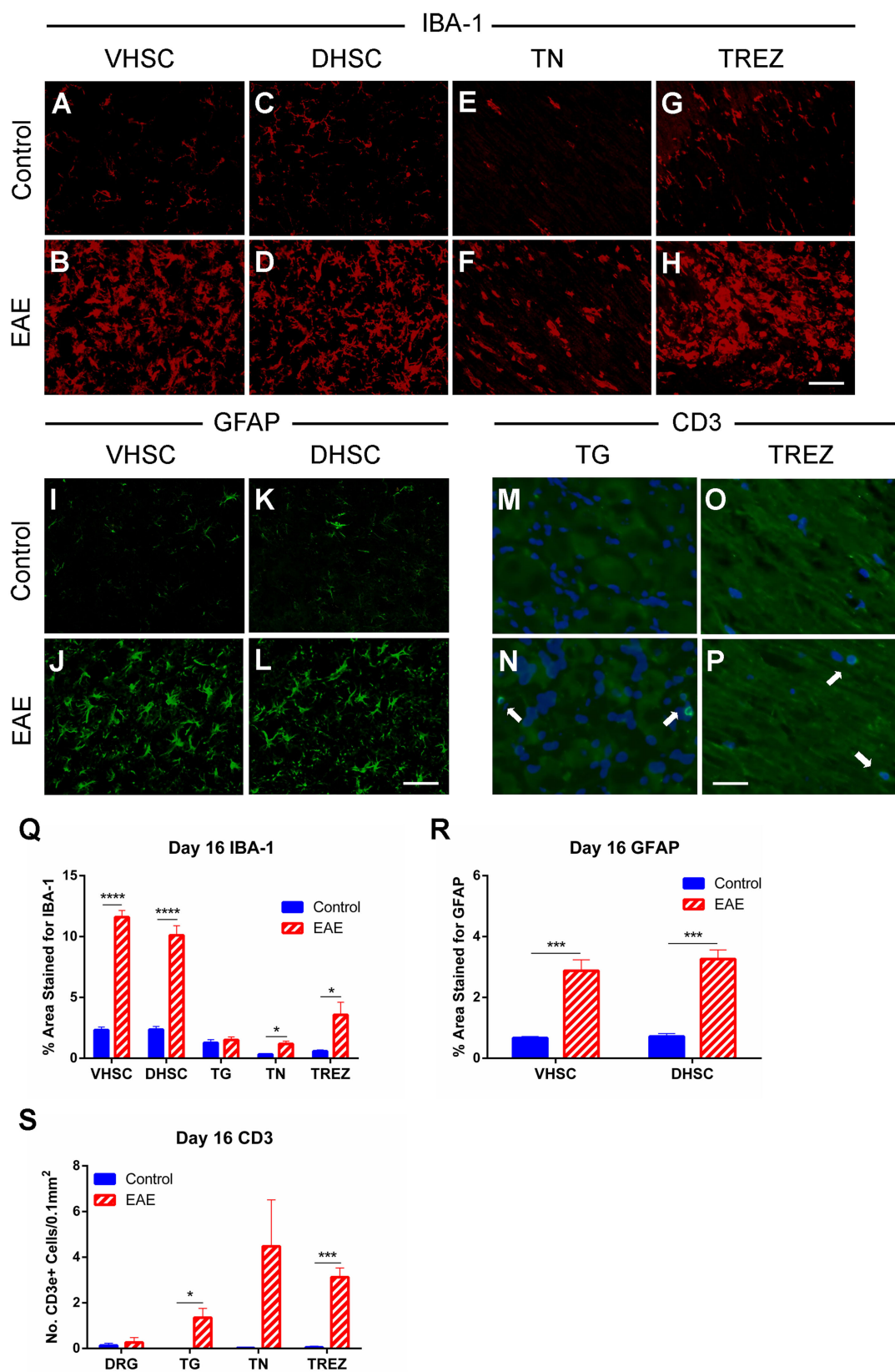


FIGURE 6 | Macrophage and glial activation and T cell infiltration in clinical EAE.

(Continued)

FIGURE 6 | Continued

IBA-1 and GFAP immunohistochemistry in the L3–5 spinal cord (SC), IBA-1, and CD3 immunohistochemistry in the trigeminal afferent pathway and CD3 immunohistochemistry in the L3–5 dorsal root ganglia (DRG) were carried out on day 16 (at the EAE clinical peak) in EAE and control mice. Representative images showing less IBA-1 immunostaining in the ventral horn of the spinal cord (VHSC) (A), dorsal horn of the spinal cord (DHSC) (C), trigeminal nerve (TN) (E), and trigeminal root entry zone (TREZ) (G) of control mice compared to the VHSC (B), DHSC (D), TN (F), and TREZ (H) of EAE mice. Representative images showing less GFAP immunostaining in the VHSC (I) and DHSC (K) of control mice compared to VHSC (J) and DHSC (L) of EAE mice. Representative images showing an absence of CD3+ cells in the trigeminal ganglia (TG) (M) and TREZ (O) of control mice, which were visible in TG (N) and TREZ (P) of EAE mice. Scale bar equals 25 μ m and white arrows indicate CD3 immunoreactive cells with DAPI nuclear staining. (Q) Bar graph showing significantly increased levels of IBA-1 immunostaining in the VHSC, DHSC, TN, and TREZ in EAE mice compared to control mice. No difference was seen in the TG between experimental groups. (R) Bar graph showing significantly increased levels of GFAP immunostaining in both the VHSC and DHSC in EAE mice compared to control mice. (S) Bar graph showing no difference in CD3+ cell numbers in the DRG, but significant increases in the TG and TREZ, as well as a trend for increased levels in the TN ($p = 0.095$) in EAE compared to control mice. * $P < 0.05$, *** $P < 0.001$, and **** $P < 0.0001$, unpaired student's t -test, $n = 4$ –5. Data are expressed as mean \pm SEM.

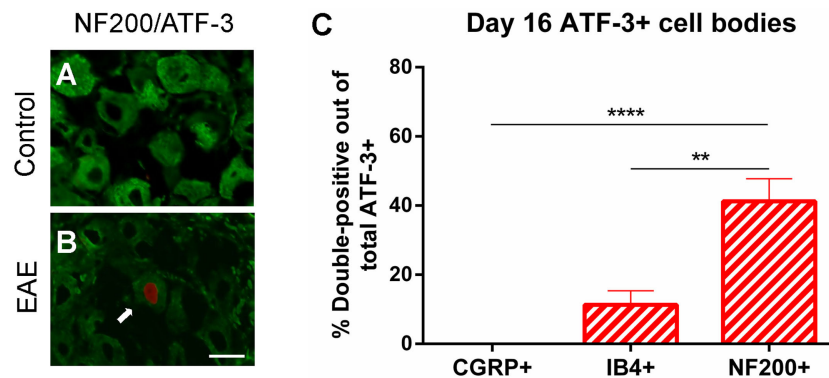


FIGURE 7 | Specific damage to myelinated A-class fibers in clinical EAE. Representative images of NF200/ATF-3 double-labeling showing the presence of NF200+ATF-3+ cells in the trigeminal ganglion of EAE mice (B), but not control mice (A) at day 16. Scale bar equals 25 μ m and white arrow indicates an NF200 immunoreactive cell with ATF-3 nuclear staining. (C) Bar graph showing significantly increased numbers of NF200+ATF-3+ cell bodies compared to CGRP+ATF-3+ and IB4+ATF-3+ cell bodies in the trigeminal ganglia of EAE mice. ** $P < 0.01$ and **** $P < 0.0001$, one-way ANOVA followed by Tukey's *post hoc* test, $n = 4$ –5. Data are expressed as mean \pm SEM.

Since previous studies have purported links between central lesions in areas such as the SC (9, 10, 12, 36) and trigeminal nuclei (15) with evoked pain behaviors, we next analyzed the CNS of mice with preclinical EAE for T cell infiltration and glial changes. No increase in CD4+ cells was observed in the brain or SC in the preclinical period, which suggests that a central adaptive immune response does not mediate allodynia in preclinical EAE. Glial activation in the dorsal horn of the SC and STN was also analyzed, and a subtle, yet statistically significant, increase in IBA-1-expressing cells in the dorsal horn of the SC was seen. Increased numbers of IBA-1+ cells have previously been shown in the primary somatosensory cortex in preclinical EAE (16), and perivascular microglial clustering in the SC occurs prior to the onset of clinical symptoms and demyelination. This clustering was shown to correlate with fibrinogen deposition in the SC, which was taken as a reflection of early blood–brain barrier disruption (37). It is unknown whether the increase in IBA-1 expressing cells in the dorsal horn was provoked by perivascular microglial clustering in the present study, and the use of chimeric mice (38–41) or a marker specific for microglia (42) would allow for clarification. Microglia have been linked to the initiation of mechanical allodynia in a range of chronic pain models (43–47). In particular, after nerve damage, spinal microglia have been shown to change their morphology, phenotype, and motility, express intracellular signaling molecules (for example, p38 mitogen-activated protein

kinase), and release brain-derived neurotrophic factor, cytokines such as tumor necrosis factor (TNF) and interleukin (IL)-1 β , and chemokines, thus potentiating aberrant nociceptive signaling (48–50). It is therefore feasible that microglial activation in the L3–5 dorsal horn may have contributed to the mechanical allodynia of the hind paw seen in this study. However, a similar increase in IBA-1 expressing cells was not apparent in the STN where small diameter fibers (C and A δ) carrying information concerning pain and temperature terminate. A more comprehensive analysis inclusive of all brainstem trigeminal nuclei would be useful to elucidate whether an increase in IBA-1+ cells similar to what is seen in the dorsal horn of the SC is apparent in these areas, and whether this may play a role in the development of facial allodynia in preclinical EAE. It is also interesting to note that different immune cells have recently been shown to mediate neuropathic pain behavior following peripheral nerve injury in male and female mice. While spinal microglia mediated the development of mechanical pain hypersensitivity in male mice, T cells appeared to be responsible for such pain in female mice (51) suggesting sexual dimorphism in pain processing.

Given that allodynia appears to arise in the absence of distinct central lesion formation, we next sought to ascertain whether changes in the PNS are associated with stimulus-evoked pain in EAE. In analyzing the trigeminal afferent pathway during preclinical EAE (day 8), we found that facial allodynia coincided

with T cell infiltration into the TN. Considering we studied MOG_{35–55}-induced EAE, the antigen specificity of the T cells infiltrating the TN, where the MOG antigen is not expressed, remains unknown. The clinical peak of EAE is associated with central neuroinflammation. T cells are known to infiltrate into the CNS (52) and trigeminal afferent pathway including the TG, TN, and TREZ (15) in EAE, and this was confirmed in the present study. Infiltrating CD4⁺ cells in EAE have previously been shown to be pathogenic and primarily of a Th1 and Th17 phenotype (53, 54), both of which have been implicated in the generation of neuropathic pain through the production of their signature cytokines IFN- γ (55, 56) and IL-17 (57, 58). T cell infiltration along with colocalized glial activation in the superficial dorsal horn (10) and trigeminal afferent pathway (15) were postulated to contribute to the development of EAE-induced evoked hind paw and facial hypersensitivity, respectively. Although pain hypersensitivity developed prior to significant central neuroinflammation, our observation of a substantial infiltration of CD4⁺ cells in the CNS, microglia, and astrocyte activation in the dorsal horn of the SC, and an increased T cell infiltration, and macrophage/microglial activation in multiple areas along the trigeminal afferent pathway may have contributed to pain behaviors during EAE clinical peak. In parallel with another recent study (17), we found increased numbers of infiltrating CD4⁺ cells in the DRG in clinical EAE, where it has been previously reported that rats with EAE have elevated expression of fractalkine (CX3CL1) and its receptor (CX3CR1) (59), and increased levels of TNF- α (60, 61), and IL-1 β (62). Innate immune cells such as NK cells, neutrophils, and mast cells are also known to be involved in the pathogenesis of EAE (63–65); however, their role in pain associated with EAE and MS is unclear. Mast cells in particular have been implicated in the production of neuropathic pain in models of peripheral nerve injury (66) and chemotherapy-induced peripheral neuropathy (67). An expanded characterization of cells such as these in the EAE model and their relationship to pain behaviors would be an important addition to the current knowledge.

The facial allodynia seen in the clinical period of EAE was associated with neuronal damage in myelinated A-class sensory fibers, but not in unmyelinated C-fibers in the TG. ATF-3 expression has also been recently shown in a mixed population of sensory neurons in EAE, including those with myelinated axons (NF200⁺) and in others that express TRPV1 (17). Previous studies in models of monoarthritic joint pain (68) and capsaicin-induced pain (69) have shown that damage to cell bodies in the DRG, as indicated by ATF-3 staining, primarily involves CGRP⁺ peptidergic and IB4⁺ non-peptidergic C-fibers. EAE is a demyelinating disease, and this could account for why myelinated sensory fibers are injured while C-fibers appear largely preserved. Large myelinated A β fibers are implicated in the dorsal horn circuit responsible for producing mechanical allodynia (70, 71). Indeed, blockade of large diameter NF200⁺ A-class sensory fibers, but not C-fibers, abrogates mechanical allodynia in a range of neuropathic pain models including chemotherapy-induced peripheral neuropathy, nerve injury, and diabetic neuropathy (72). Further studies addressing whether A β fibers are specifically damaged in

EAE, and whether this occurs in the dorsal horn circuit as well as the trigeminal afferent pathway would help to elucidate the etiology of allodynia in MS.

The observation that the facial allodynia and grimacing observed in the current study predominate in unique clinical phases of EAE is an interesting one. Facial allodynia develops in the absence of overt central changes but rather corresponds with a subtle peripheral change of a small, yet significant, infiltration of T cells into the TN. Facial allodynia appears to quite sharply decrease following the onset of EAE compared to the preclinical period. While it is presently unknown why this occurs, hypoesthesia is also common in MS (35), and diminished responses to subcutaneous formalin injection in EAE have been linked to dysregulation of the glutamatergic system (73). The mechanisms underlying the facial grimacing observed in clinical EAE are likely to be multifactorial as the behavior coincides with T cell infiltration into the CNS, trigeminal afferent pathway and DRG, as well as an increase in IBA-1⁺ cell numbers and specific damage to large myelinated neurons in the trigeminal afferent pathway, gliosis in the dorsal horn of the SC, and central demyelination. Although the precise etiology of facial allodynia and grimacing in EAE remains unclear, both methods provide an invaluable approach to better understand the mechanisms involved in the production of pain in EAE.

In summary, we observed different pain phenotypes including stimulus-evoked and spontaneous pain predominating in unique stages of the chronic EAE model, in accordance with the diverse pain phenotypes seen in MS patients (6). The pain behaviors we observed were associated with several neuroinflammatory changes in both the peripheral nervous system and CNS and are likely to involve numerous underlying mechanisms.

AUTHOR CONTRIBUTIONS

SD designed experiments, performed animal immunizations, behavioral tests, flow cytometry, immunohistochemistry, microscopy, image analysis, and drafted the manuscript. CP assisted with behavioral testing and immunohistochemistry. PM was involved in tissue dissection and assisted with image analysis. JL and PC assisted in experiment design and interpretation of data. GM-T conceived and designed the study, assisted in interpretation of data, and critically revised the manuscript. All authors read and approved the manuscript.

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Developmental Changes in Pain and Spinal Immune Gene Expression after Radicular Trauma in the Rat

Gordon A. Barr^{1*}, Shaoning Wang¹, Christine L. Weisshaar² and Beth A. Winkelstein²

¹ Division of Basic Science Research, Department of Anesthesiology and Critical Care Medicine, The Children's Hospital of Philadelphia, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA, USA, ² Spine Pain Research Laboratory, Department of Bioengineering, School of Engineering and Applied Science, University of Pennsylvania, Philadelphia, PA, USA

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*Correspondence:

Gordon A. Barr
barrg@email.chop.edu

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Neuropathic pain is chronic pain that develops after nerve injury and is less frequent in infants and children than in adults. Likewise, in animal models of neuropathic pain, allodynia and hyperalgesia are non-existent or attenuated in the infant, with a “switch” during development by which acute nerve injury transitions to chronic pain. Concomitant with the delay in neuropathic pain, there is a parallel delay in the ability of nerve injury to activate the immune system. Models of neuropathic pain in the infant have used various ligation methods and find that neuropathic pain does not occur under after postnatal days 21–28 (PN21–PN28), linked to activation of immune processes and developmental regulation of anti-inflammatory cytokines. We applied a model of neuropathic pain in the adult using a transient compression of the cervical nerve or nerve root in infant rats (injured at 10, 14, 21, or 28 days of age) to define transition periods during which injury results in no change in thermal and mechanical pain sensitivity or in short-term changes in pain. There was little to no hyperalgesia when the injury was imposed at PN10, but significant thermal hyperalgesia and mechanical allodynia 1 day after compression injury when performed at PN14, 21, or 28. Thermal withdrawal latencies returned to near baseline by 7 days postsurgery when the injuries were at PN14, and lasted up to 14 days when the injury was imposed at PN28. There was mechanical allodynia following injury at 1 day postinjury and at 14 days after injury at PN14. Measurements of mRNA from spinal cord at 1, 7, and 14 days postinjury at PN14, 21, and 28 showed that both the magnitude and duration of elevated immune markers and chemokines/cytokines were greater in the older animals, corresponding to the development of hyperalgesia. Thus, we confirm the late onset of neuropathic pain but found no evidence of emergent hyperalgesia if the injury was before PN21. This may be due to the use of a transient, and not sustained, compression ligation model.

Keywords: neuropathic pain, compression, ontogeny, immune, cytokines, chemokines, hyperalgesia, allodynia

INTRODUCTION

As with all sensory systems, nociceptive circuits are plastic in infancy. Neonates respond to acute painful stimulation and show hyperalgesia after inflammatory injury at or before birth (1–6). Nonetheless nociceptive circuits and resultant pain processes develop and change well into postnatal life (1, 7–13). Neuropathic pain following peripheral nerve damage is one example of a process that

is immature in the infant. Phantom limb pain exists in children, but the incidence is 10-fold less than for adults. Complex regional pain syndrome is rare until adolescence. Brachial plexus avulsion often produces severe and debilitating pain in adults but not when it occurs obstetrically (14–16). Similarly in the rodent infant, regardless of the injury model or the age at which the injury occurs, changes in pain thresholds appear only when tested at 21–33 days of age [(17–27); see **Table 1**]. In some studies, there was a delay between injury and decreased thresholds or an earlier resolution of the pain (19, 21, 27). Thus the increased propensity to develop neuropathic pain does not appear until early adolescence in both the rat and human [reviewed in Ref. (14–16, 18)].

There are multiple mechanisms that have been proposed to be responsible for neuropathic pain [see Ref. (18, 28–36) for examples and reviews]. Injury activates both the innate (37–41) and adaptive immune (17, 41–44) systems and dampening the immune response reduces pain (45, 46). Thus, the immune system is involved in the initiation and maintenance of neuropathic pain following peripheral or central nervous system damage (29, 47). These immune processes may maintain neuropathic pain, which are then amenable to immune suppressant drugs. The mechanisms that induce and maintain neuropathic pain that are immature in the infant, or that may be protective, are largely unknown, although recently two inhibitory cytokines, IL-4 and

TABLE 1 | Summary of published peripheral nerve injury studies on pain during development.

Model	Age	Short-term pain	Long-term pain	Mechanism	Reference
• Caudal trunk transection	PN0 PN10 Adult	• No effect for PN0 or PN10 • Immediate for adult	• Appeared at 4–6 weeks postinjury in PN0 and PN10		(21)
• C-fiber stimulation	PN10 Adult	• Allodynia 3–48 h in adult but not PN10	• Not tested	• Spinal microglia activation only in adult	(23)
• L5, L6 ligation	PN7 PN14 PN21	• Appeared when tested at PN21 regardless of age of lesion	• Resolved at 6–8 weeks for PN7 and PN14 but not PN21		(19)
• Partial sciatic (Seltzer) • Spinal nerve (Chung)	PN14 PN28 Adult	• PSL – no effect at PN14 • SNL – all ages allodynia 1 week postinjury (PN21, PN35)	• PN14 resolved 4–6 weeks • PN28 resolved 7 weeks • Adult resolved 8 weeks		(27)
• Spared nerve injury (SNI) • Chr. constriction (PN10 only)	PN3 PN10 PN21 PN28 PN33	• SNI-PN3 no effect • PN10 and 21 show non-specific transient allodynia. 7 days postop • PN33 allodynic	• Only PN33 shows long-term effect • No reappearance		(20)
• SNI • i.t. NMDA, LPS, or activated microglia	PN10 PN21	• Not tested for SNI • LPS produces small but significant allodynia in PN10 and PN21 • Activated microglia had no effect	• Not tested	• Spinal microglial markers less elevated at PN10 SNI • NMDA and LPS elevated microglial markers at both ages	(22)
• SNI • i.t. LPS • i.t. ATP activated microglia	PN3 PN10 PN21 Adult	• Not reported	• Not tested	• Adult spinal microglia (3 days) and astrocyte (5 days) activation • Infant microglia weak but early (1 day) robust astrocyte activation	(24)
• SNI	PN10 Adult	• No immediate allodynia at PN10	• Not tested	• Genes related to immune function activated only in adult DRG • Macrophages cluster around A-fiber cell bodies only in adult	(25)
• SNI	PN10 Adult	• No allodynia before PN21	• Appeared only at PN33 after PN10 injury	• T-cells infiltrate the spinal cord in adults not infants • Identify different genes expressed in adults vs. infants related to immune response	(17)
• SNI	PN10 PN35 Adults	• No immediate allodynia at PN10	• Cold, mechanical, and weight bearing changes only after PN30. No thermal changes	• Upreg. of selective immune markers • Anti-inflammatory IL-4 and IL-10 cytokines are protective in infants	(48)
• SNI • Minocycline or ketamine treatment	PN10	• No immediate allodynia at PN10	• Mechanical allodynia only after PN31. No thermal changes	• Allodynia accompanied by macrophage, microglial, and astrocyte activation • NMDA dependent	(72)

IL-10 were overexpressed in the infant and protective, inhibiting neuropathic pain [(48); reviewed in Ref. (18)]. Concurrent with the lack of neuropathic pain is the limited ability of nerve injury to activate immune markers in the spinal cord or DRG (19–22, 24, 25, 27). We do not fully know why the immune response to nerve damage is immature. Immune activation in neonates can be induced by other insults (e.g., ischemic brain injury; i.t. NMDA, *Escherichia coli* or LPS injection; and intraplantar carrageenan injection). It is not therefore, the inability of the immune system to respond but rather the inability of neural injury to activate the immune system.

The specific goal here is to understand the mechanisms that protect the infant from developing chronic neuropathic pain and how those mechanisms change as the infant matures. Studies of neuropathic pain in the infant have focused on peripheral nerve injury using a variety of models, almost all of which use some form of permanent/sustained nerve ligation. We have adapted rat models of transient cervical nerve or root compression and applied them to infants (**Figure 1A**). This avoids potential interactions of ligation with nerve growth during early development and has not been tested in infants. In adult rodents, cervical root compression, a transient one-time event, induces long-term thermal and mechanical hyperalgesia (49, 50). In humans, the comparable injury is brachial plexus avulsion. To test the maturation of the behavioral and immune

responses to this more transient injury, we induced the compression injury at 10–28 days of age and assessed pain responses 1, 7, and 14 days later to bracket ages at which there are no effects on pain thresholds and when neuropathic pain first appears (19–27). In addition, we measured mRNA for immune related markers and cytokines at those time points. We found that the injury produced no neuropathic pain in infants, short-term allodynia, and hyperalgesia that resolves quickly in weanlings and a longer lasting change in thermal pain thresholds in juveniles. Furthermore, the number, intensity, and duration of immune mRNA responses induced by injury increased with age and were greater following root injury than nerve injury. Our working hypotheses were that (1) nerve root compression activates the immune system, which is necessary but not sufficient to produce long-term pain; and (2) this activation follows the developmental course of neuropathic pain.

MATERIALS AND METHODS

Subjects

Subjects were male and female Long–Evans rats from Harlan Labs (now Envigo) born in the CHOP colony maintained at 21°C with a 12:12 light cycle. Food and water were available *ad libitum*. Cages were checked twice daily at 10 a.m. and 6 p.m.

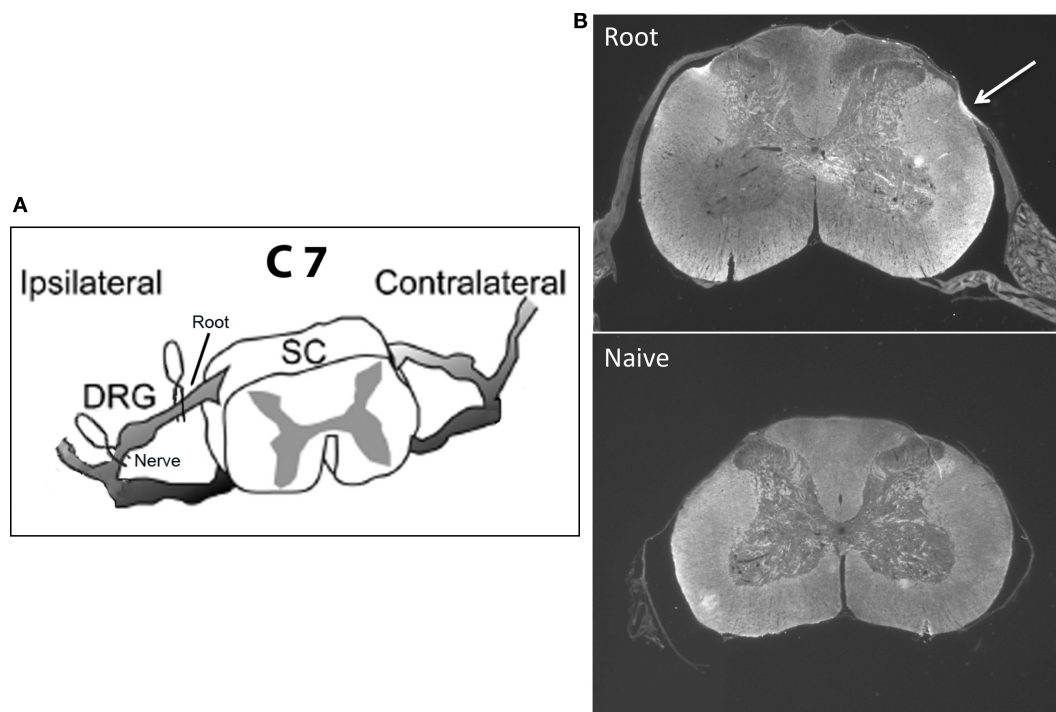


FIGURE 1 | (A) shows a schematic of the location of the microclips distal (nerve compression) and proximal (root compression) to the DRG. The nerve root compression was applied to the dorsal root as it exited the DRG. The nerve compression was of the mixed nerve just prior to its entering the DRG. **(B)** is a sample photomicrograph of myelin basic protein staining 14 days following root compression in a 28-day old animal (top) and a same age control (bottom). The arrow denotes the site of compression. We use these figures only to show the nature of the compression injury and not to imply any quantitative changes.

and the day of birth was defined as PN0. Pups were weaned at PN21, after behavior testing, and housed 2–3/cage for the remainder of the experiment. The Institutional Animal Care and Use Committees at CHOP approved all animal care and experimental procedures, which follow the guidelines from the National Institutes of Health.

Experimental Design

Experimental groups were always within a single litter and included: central nerve root compression; peripheral nerve compression, sham operated and naïve controls. Testing experimental groups within litters greatly reduces variability and increases power. No more than one condition was repeated in a single litter for any dependent measure. Surgery was performed on 10-, 14-, 21-, or 28-day old animals. We tested for thermal hyperalgesia and tactile allodynia 1, 7, and 14 days postsurgery (PS1, PS7, and PS14) in the same animals, except for the PN10 animals who showed no or minimum pain at either 1 or 7 days and were not tested further. At each postoperative time point other animals were sacrificed for either immunohistochemistry or quantitative RT-PCR. We were not powered for rigorously testing sex differences, but preliminary analyses showed no differences for either thermal and mechanical behaviors. For PCR, the smaller *N* and uneven distribution of males and females made those comparisons impossible. Therefore, for all subsequent analyses male and female data were combined.

Our primary interest was root compression for comparison to the adult literature [Figure 1A; (50–52)]. We also tested nerve compression because with few exceptions the infant methods have damaged a nerve. Data for the sham root and sham nerve surgeries were very similar. Therefore, to reduce animal numbers, in some litters, a single root or nerve sham surgery was performed rather than both, and the data combined. A separate untreated group served as the non-surgical naïve controls, and withdrawal latencies for the left and right paws were averaged for those controls.

Surgery

Compression was conducted in fully anesthetized rats aged 10, 14, 21, or 28 days of age. These ages include the human equivalents of newborns through early adolescence (Table 2). All surgical procedures were performed under inhalation anesthesia (4% induction with isoflurane and 2% isoflurane maintenance with oxygen) using aseptic techniques. Breathing rate and hindpaw pinch response were monitored throughout surgery to ensure adequate depth of anesthesia. Rodents were placed in a prone position and any hair on the back of the neck

was removed, and the area disinfected using Betadine, followed by alcohol and then covered with a sterile drape. Following a skin incision using a sterile scalpel, the right paraspinal muscles were separated from the spinous processes at the C4–T2 levels. The laminae, facet joint, and spinous process on the right side at C6–C7 were carefully exposed under a surgical microscope. The right transverse process was removed at the C7 level to expose the C7 nerve roots of interest. Central or peripheral nerve compression was *via* surgical vessel microclips and was unilaterally applied to the C7 right mixed nerve or nerve root (Figure 1A). Compression was applied for 15 min and then removed. Microclips have been commonly used in other animal models of radiculopathy (49, 50, 53). Sham procedures were performed in separate groups of rats and involved all surgical procedures except that the nerve or root was not disturbed. After surgery, all wounds were washed with preservative-free sterile saline and closed in two layers (paravertebral muscle layer and skin) with monofilament suture and surgical glue. For the 10- and 14-day old pups, a commercially available nail biter/thumb sucking deterrent was placed near the surgical region to discourage the mother from disturbing the wound. Rats recovered in a cage warmed by a heating pad in room air and closely monitored by the surgeon for up to 1 h following surgery. Typically, most rats recovered from the effects of surgery within 10–20 min. This study of pain precluded the postlesion use of analgesics in all groups. The rats were monitored daily following surgery and did not exhibit any signs of distress or infection.

Tests for Thermal Hyperalgesia

Thermal withdrawal latencies were assayed under non-restrained conditions for both forepaws. Each animal was placed singly within an inverted Plexiglas cage upon an elevated glass pane maintained at 30°C (e.g., Hargreaves apparatus). A radiant heat source (24 V halogen lamp focused through a convex lens to a 2 × 4 mm area) beneath the glass was trained on the lateral plantar surface of the forepaw. The heat stimulus and an automatic timer were activated simultaneously. When the rat lifted its paw, the switch simultaneously turned off the stimulus and timer. Cut-off time was set at 20 s to avoid tissue injury.

Tests for Mechanical Allodynia

The tactile sensitivity for both forepaws was measured as the latency to withdraw the paw to a mechanical probe. Each rat was previously acclimated to the environment and experimenter and gently restrained. In each session, a series of tactile stimuli were placed on the dorsal surface of each forepaw, just behind the interdigital web between the second and third toes. We used differing weighted probes that floated freely [3.3, 5.6, 7.5, 10.1 g; (54, 55)]. Latency to move the paw was measured. Because there were few interactions of the treatment with intensity, we averaged the response latency over the intensities. For all testing procedures, animals were free to remove themselves from the stimulus.

Dissection

Following testing, subjects were deeply anesthetized with a sodium pentobarbital and when fully unresponsive to pinches

TABLE 2 | Rough age equivalents between rats and humans.

Rat	Human
PN10	Newborn
PN14	Early childhood
PN21	Early preadolescent child
PN28	Early adolescent, presexual maturity

PN is postnatal day.

and air puffs, they were either transcardially perfused (4% paraformaldehyde) for IHC or decapitated for qPCR. Following perfusion, the spinal cord was dissected out and placed overnight in paraformaldehyde. The cervical cord, just rostral to the compression site, was blocked and placed in sucrose prior to cryostat sectioning. For qPCR, the spinal cord was rapidly removed and placed on ice. The cervical enlargement was isolated, and the dorsal cord above the central canal (separate for the ipsilateral and contralateral sides to the compression side) was removed, and frozen at -80°C until assayed.

Quantitative RT-PCR

We assayed c-fos and a number of markers of immune related function, chemokines, and cytokines by quantitative RT-PCR (Taqman) in the dorsal spinal cord 1, 7, and 14 days postsurgery (except at PN10) in parallel with the behavioral studies. Each was chosen as a marker of immune related processes such as cyclooxygenase 2 (COX-2), toll-like receptor 4 (TLR-4), ionized calcium-binding adapter molecule 1 (IBA-1; for microglia) or Glial fibrillary acidic protein (GFAP; for astrocytes), or because they were inflammatory or anti-inflammatory cytokines and chemokines that have been implicated in chronic or neuropathic pain (see **Table 3**). Standard methods were used. Briefly, after homogenizing with guanidine-thiocyanate-containing lysis buffer, total RNA was isolated using RNeasy Mini Kit (Qiagen). Traces of DNA are then removed by DNase treatment on the column and the total RNA was eluted in RNase-free water. Five microgram DNase-treated total RNA from each sample was incubated with 50 pmol T7 (dT)24 primers (Affymetrix) at 70°C for 10 min. Using this RNA as a template, single-strand cDNA was synthesized by incubating with Reverse Transcriptase (SuperScript II RT, Invitrogen) as well as first Strand Buffer and 10mM dNTP (Invitrogen) at 42°C for 1 h followed by 70°C for 15 min to denature the enzymes. Newly synthesized ss-cDNA was diluted in pure H_2O . Real-Time PCR was performed using

a StepOne plus machine with TaqMan fast advanced master mix and Taqman primers (all Applied Biosystem), using the “Fast” protocol. The exceptions were IL-4 and IL-10 which were assayed by SYBR Green methods as previously described (56). To quantitate the mRNA changes, we used the $\Delta\Delta\text{Ct}$ method with GADPH as the reference primer. We have compared GADPH to S18 and β -actin and found identical results previously.

IHC

We stained 30 μm frozen floating sections for myelin basic protein 1 (Covalence) at a dilution of 1:1,000 using standard ABC protocols (57) as described previously (58). Controls include staining without the primary antibody. We did not quantitate the resulting micrographs.

Statistical Analysis

For the behaviors, withdrawal latencies were analyzed by two-way ANOVAs to determine overall significant differences. The two factors were lesion type (ipsilateral to compression, contralateral to compression, ipsilateral sham, and naïve animals) and days postsurgery. Because all surgeries were within litters and each animal was tested at each time point, both factors were considered matched values and analyzed by a repeated-measures ANOVA. Tukey's multiple comparison tests were used to determine individual comparisons of the ipsilateral compression subjects to the naïve animals and to the ipsilateral sham controls. We did not compare the ipsilateral to the contralateral side because in some cases the contralateral side was altered as well, although not significantly. Those data are shown but not used in the statistical comparisons.

qPCR

The number of cycles for the naïve group (compared to GADPH) was subtracted from each experimental condition in the same litter and analyzed by a two-way ANOVA. Because all treatments were in a single litter, that analysis was repeated measures. Each time point was analyzed separately and Tukey tests for multiple comparisons were between the injured ipsilateral side and the ipsilateral sham control. The data are presented as fold change relative to the naïve animals.

RESULTS

Histology

Neither root nor nerve compression induced gross qualitative changes in the dorsal horn structure (**Figure 1B**). We did not quantitate these results and thus cannot make conclusions about relative changes in myelin or cell density at different ages and times postinjury.

Behavioral Tests

Figures 2 and 3 show the results of root or nerve compression on the thermal response latency. Comparisons were of the ipsilateral paw of the compressed root or compressed nerve to the ipsilateral paw sham and the naïve controls. In separate analyses, the contralateral side did not differ from either the

TABLE 3 | Function of immune markers from Figures 4 and 5.

IBA-1	Marker for macrophage/microglia activation
GFAP	Marker for astrocytes
COX-2	Inducible enzyme for production of prostaglandins
TLR-4	Receptor for gram-negative bacteria (LPS); activates innate immune system
IL-1 α	Proinflammatory cytokine
IL-1 β	Proinflammatory cytokine
IL-4	Anti-inflammatory cytokine
IL-6	Proinflammatory cytokine
IL-10	Anti-inflammatory cytokine
CCL2 (MCP-1)	Proinflammatory chemokine
CCL3 (MIP-1a)	Proinflammatory chemokine
CCL5 (Rantes)	Proinflammatory chemokine
TNF α	Proinflammatory cytokine

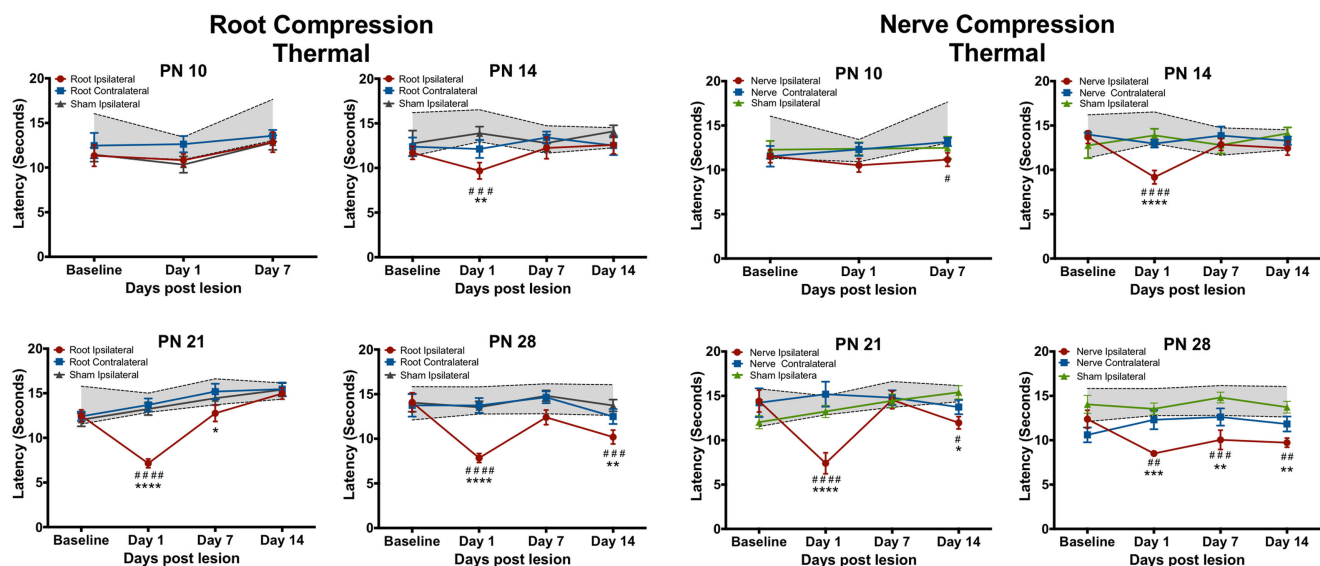


FIGURE 2 | The change in the forepaw response to the thermal stimulus following root or nerve compression. The age of surgery is above each panel and the X-axis denotes the time of testing postsurgery. The 95% confidence intervals for the naïve controls are denoted by the shaded area between the dashed lines. Significant differences from the naïve subjects are denoted by * and from the forepaw ipsilateral to the sham surgery by #. $N = 8$ except PN14, $N = 6$.
*/# $p < 0.05$; **/# $p < 0.01$; ***/### $p < 0.001$; ****/#### $p < 0.0001$.

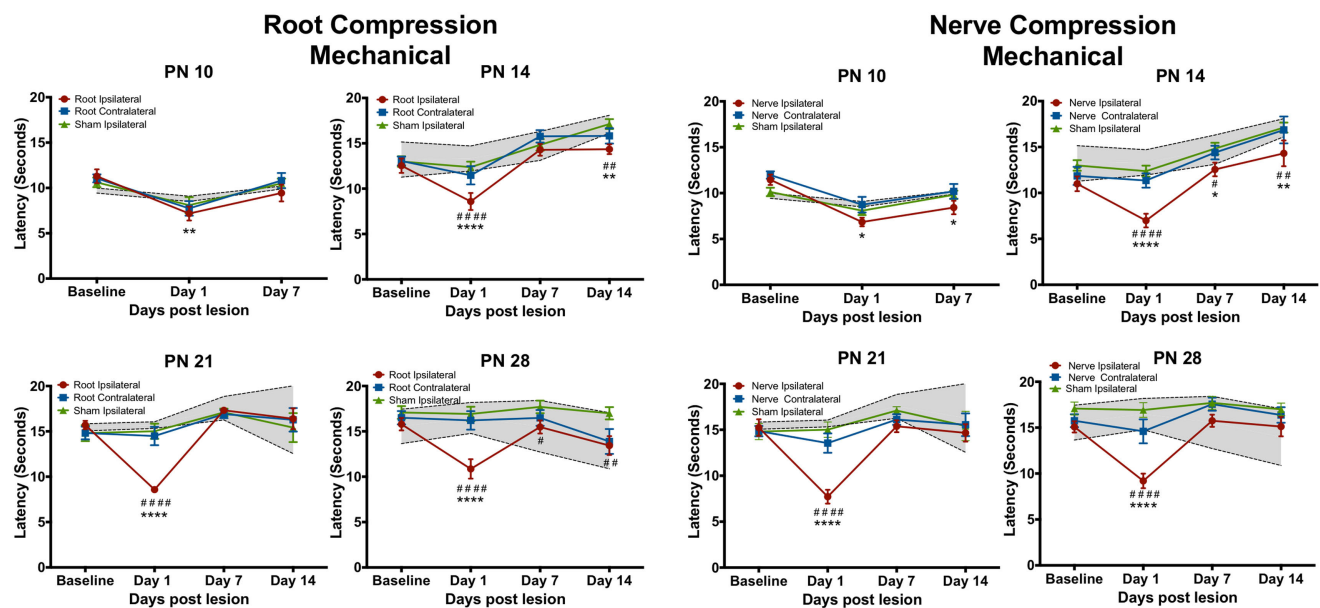


FIGURE 3 | The change in the forepaw response to the mechanical stimulus following root or nerve compression. Details are as in Figure 2. $N = 8$ for PN10 and PN28; $N = 6$ for PN14 and PN21.

sham contralateral side or the naïve controls for the thermal test and only at one comparison for the mechanical test (PN14, PS1). F -values presented below are for the treatment \times post-surgical day interactions. Multiple comparison p -values are in Figures 2 and 3.

Thermal Tests

Neither root nor nerve compression at PN10 changed pain responses compared to the sham control [Root: $F(6,42) = 0.731$, $p = 0.627$; Nerve: $F(6,42) = 0.998$, $p = 0.439$]. However, at PN14, PN21, and PN28, both nerve and root injuries produced a large

reduction in the thermal response latency 1 day postsurgery [Root/PN14: $F(9,45) = 1.489$, $p = 0.181$; PN21: $F(9,63) = 6.84$, $p < 0.0001$; PN28: $F(9,63) = 3.691$, $p = 0.001$; Nerve/PN14: $F(9,45) = 3.237$, $p = 0.004$; PN21: $F(9,36) = 5.765$, $p < 0.001$; PN28: $F(9,45) = 1.982$, $p = 0.064$]. At PN14, withdrawal latencies following root or nerve injury returned to control levels 7 and 14 days postsurgery. At PN21, the root injuries reduced pain thresholds 1 day postoperatively, returning to control levels at PS7 and PS14. Nerve compression at PN21 showed a slightly different result with a return to control levels at PS7 but a reappearing slight hyperalgesia at PS14. When either nerve or root compression was performed on PN28, the hyperalgesia lasted for 14 days after surgery, the longest time tested.

Mechanical Tests

The mechanical allodynia results differed slightly from those of the thermal test. In separate analyses, the contralateral side never differed from either the sham contralateral side or the naïve controls, except at PN14, PS1. Again at PN10, there were no effects when compared to the ipsilateral sham control but there was a slight reduction (< 2 s) in latency compared to naïve controls for both root and nerve injury [Root: $F(6,42) = 3.327$, $p = 0.010$; Nerve: $F(6,42) = 4.491$, $p = 0.001$]. At PN14, 21, and 28, there was a short-term increase in sensitivity at PS1 [Root/PN14: $F(9,45) = 5.315$, $p = 0.002$; PN21: $F(9,36) = 9.915$, $p < 0.0001$; PN28: $F(9,63) = 5.425$, $p < 0.001$, $p = 0.001$; Nerve/PN14: $F(9,45) = 2.756$, $p = 0.012$; PN21: $F(9,36) = 6.056$, $p < 0.001$; PN28: $F(9,63) = 5.425$, $p < 0.001$]. Following root injury, the withdrawal latencies returned to baseline levels by PS7 and PS14, regardless of the age of injury, except when performed at PN14. There was a reduction in the latency at PS14, largely because of increased latency in controls at those times that were actually higher than baseline latencies. For the nerve compression, at PN14, there was hyperalgesia at all postsurgical

times. When surgery was performed at PN21 or 28, latencies returned to control levels by PS7 and PS14.

qPCR – Root Compression

There were several patterns that emerged from these data (Table 4; Figures 4 and 5). First, the number and levels of elevated markers were highest at 1 day postsurgery. However, at PN14 and to a lesser degree at PN21, the number and magnitude declined at later time points. The magnitude of changes at PN28 declined over time but the number of significant changes remained constant. Thus, overall activation of cytokines, chemokines, and other immune markers was more prolonged the older the animal at surgery (detailed below).

At PN10, only CCL2 was significantly elevated by root compression compared to controls at PS1, and TNF α and CCL5 were significantly elevated at PS7.

By PN14, a number of proinflammatory cytokines were stimulated (IL-1 β , IL-6, CCL2, and CCL5). This begins a pattern of consistent activation for CCL2 and CCL3, which were elevated at all postsurgical ages at PN14 and older. This activation declined such that by 7 days postsurgery, there were few differences and only CCL2 and CCL3 were at the twofold change level and significantly different from controls. At PS14, the microglia marker, IBA-1 and IL-1 β were significantly elevated in addition to CCL2 and CCL3.

When injury occurred at PN21, 1 day postsurgery, there were significant changes in the IL-6, CCL2, and CCL3 at higher expression levels than at younger ages. The inflammatory chemokines CCL2 and CCL3 were expressed at 10–25-fold compared to controls. Some proinflammatory cytokines/chemokines were also elevated on the contralateral side (IL-1 β , CCL2, CCL3, and TNF α) but not to the extent of the injured side. Seven days postsurgery, IL-1 β and CCL3 were significantly different from controls and many other immune markers were increased twofold, although

TABLE 4 | Summary of qPCR results.

	PN10			PN14					PN21					PN28							
	PS1		PS7	PS1		PS7		PS14		PS1		PS7		PS14		PS1		PS7		PS14	
	R	R	N	R	R	N	R	N	R	R	N	R	N	R	R	N	R	R	N	R	N
c-fos	*																**	*			
IBA-1							*							**	^			****		*	
GFAP																	*	*			
COX-2												^					**				
TLR-4						^^						^^^	*	^		**	**	^			
IL-1α						^															
IL-1β		*		*			*	^		***		*			**						
IL-4																					
IL-6			^	****						***					****		^^				
IL-10																					
CCL2	***		^^^	****	**	^^^^	*			****					****	***	^^^^	****		^^	
CCL3				****	****	^	****	^^^		****	***		****	^^^		****	****	^	****		
CCL5		*											**			***					
TNFα												^									

R is root compression; N is nerve compression.

* $^{\wedge}$ $p < 0.05$; ** $^{\wedge}$ $p < 0.01$; *** $^{\wedge}$ $p < 0.001$; **** $^{\wedge}$ $p < 0.0001$.

Note that there are no data for nerve compression at PS1.

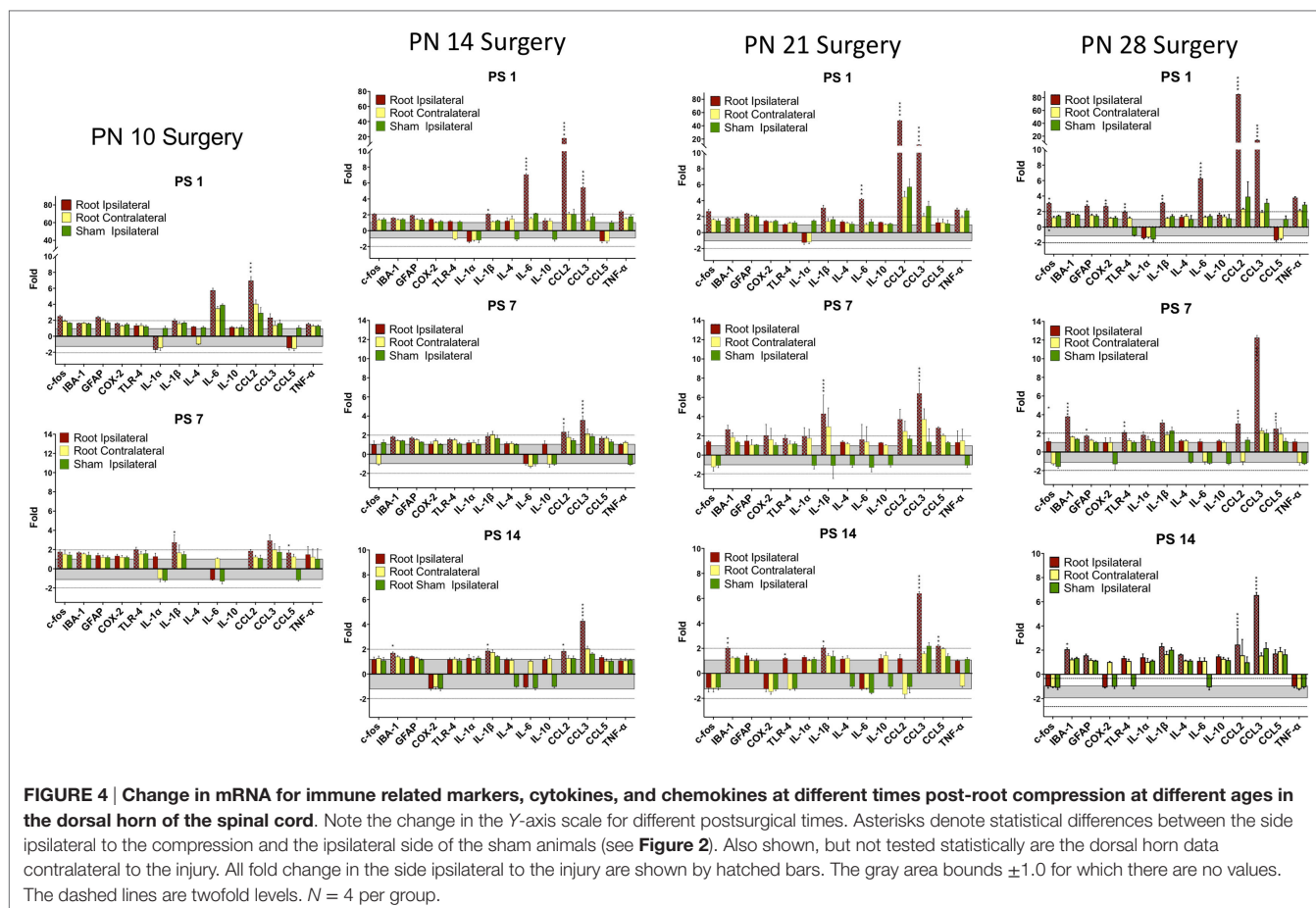


FIGURE 4 | Change in mRNA for immune related markers, cytokines, and chemokines at different times post-root compression at different ages in the dorsal horn of the spinal cord. Note the change in the Y-axis scale for different postsurgical times. Asterisks denote statistical differences between the side ipsilateral to the compression and the ipsilateral side of the sham animals (see Figure 2). Also shown, but not tested statistically are the dorsal horn data contralateral to the injury. All fold change in the side ipsilateral to the injury are shown by hatched bars. The gray area bounds ± 1.0 for which there are no values. The dashed lines are twofold levels. $N = 4$ per group.

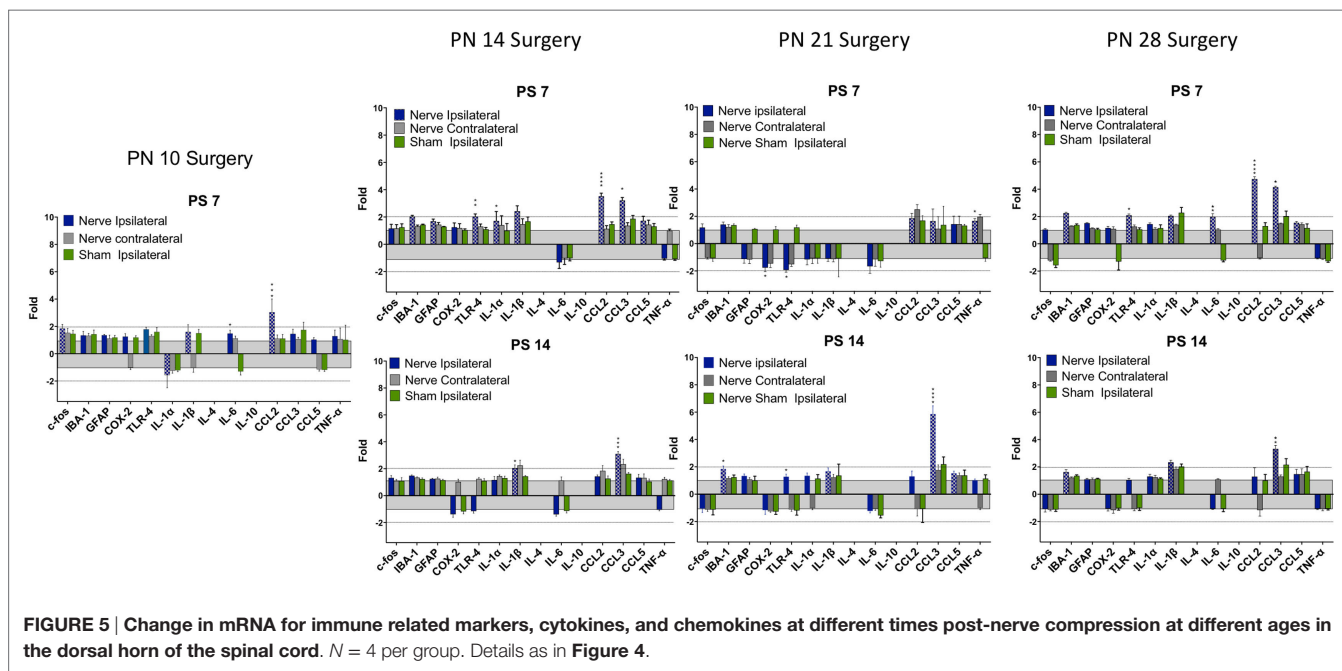


FIGURE 5 | Change in mRNA for immune related markers, cytokines, and chemokines at different times post-nerve compression at different ages in the dorsal horn of the spinal cord. $N = 4$ per group. Details as in Figure 4.

not statistically different from controls, where were also elevated. At 14 days postsurgery, there was still elevation of IBA-1, IL-1 β , and CCL3.

At PN28, 1 day postsurgery, mRNA for COX-2, FOS, GFAP, TLR4, and the proinflammatory cytokines/chemokines, including IL-1 α , IL-1 β , IL-6, CCL2, and CCL3 were all increased. At postsurgery day 7, many remained elevated and there was now increased expression of IBA-1 and GFAP. At postsurgery day 14, CCL2, CCL3, and IBA-1 remained significantly elevated. These data were consistent with those of others and confirm a late onset for immune activation following nerve injury.

There were some consistencies among those changes over time. IBA-1, the microglia marker, showed a delayed elevation at PS14 in all three of the older animals. CCL2 and IL-6, in contrast, were elevated at PS1 at the three older ages. Likewise, IL-1 β showed greater increases with age. CCL3 was elevated at all time points at PN14 and older. Only when surgery was at PN28, were c-fos, COX-2, GFAP, and TLR-4 were elevated.

qPCR – Nerve Compression

There were fewer changes at PS7 and PS14 following nerve injury than root injury and the levels were of lower magnitude, with the caveat that we did not assess changes at 1 day postsurgery. Only CCL3 was consistently elevated at PS14 at any age.

DISCUSSION

Using a single brief compression injury to either the dorsal nerve root or to the nerve, we found an immediate (1 day) thermal hyperalgesia and mechanical allodynia when injury was at 14–28 days of age but minimally at PN10. This response, 1 day after surgery, has often been reported for adult animals in multiple models [e.g., Ref. (59)]. However, consistent and prolonged thermal hyperalgesia was only seen when the surgery was at PN28. Mechanical allodynia was less prolonged lasting beyond 1 day postsurgery only at PN14. It is unclear why this occurred only at PN14, although the behavioral response to the mechanical stimulus was more variable in the older animals.

There were fewer and shorter-lasting changes in dorsal horn expression of proinflammatory cytokines/chemokines when root compression surgery was prior to PN28 with only CCL3, and to a lesser extent, CCL2, showing long lasting overexpression. However, at PN14, PN21, and PN28, IL-6 was upregulated on the first day postsurgery and returned to baseline 1 week postsurgery. Likewise, the microglial marker, IBA-1 was overexpressed at the later postsurgical times when surgery was conducted at those three ages. At PN21 and PN28, there was a particularly striking overexpression for CCL3, which showed a 40–75-fold change 1 day after surgery. At PN28, there were substantially greater numbers and levels of expression, including GFAP, TLR-4, COX-2, the interleukin cytokines, and CCL2 and CCL3. Note that IBA-1 and TLR-4 both increased when the animals were tested at 35 days of age regardless of whether the injury was at PN21 (tested at PS14) or PN28 (tested at PS7). The overexpression for most markers lasted at least 7 days, and CCL2 and CCL3 were continually expressed at high levels through the 14 days of postsurgical testing. These results are consistent with the demonstrated increased immune

cell reaction and glial response to nerve injury with age [see Ref. (18) for a recent review].

The proinflammatory cytokines and chemokines are established regulators of neuropathic pain and both mRNA and protein are elevated following nerve injury in adults. CCL2, CCL3, CCL5, IL-1 β , and IL-6 are upregulated in damaged nerves, DRGs, and spinal cord dorsal horn (60–63) in variety of models and neuropathic pain is decreased by antagonists, neutralizing antibodies, microglia inhibitors, or in CCL2 deficient mice (62, 64–66). However, in the present study, CCL3 expression was elevated at all ages at PS1 in the root compression and at PS7 in the nerve compression injury, which is not consistent with the appearance of thermal or mechanical hyperalgesia. It is possible that the early CCL2 expression is necessary but not sufficient to drive the behavioral changes.

CCL5 recruits leukocytes to a site of injury in the adult (67) and is upregulated by injury. Reduction of CCL5 by neutralizing antibodies reduces hyperalgesia. In the current experiments, CCL5 was only minimally upregulated root compression surgery at PS21 and PS28, and then only 14 or 7 days after surgery, respectively. CCL5 was not altered by nerve compression in any group. The lack of upregulation of CCL5 is consistent with at least one adult study using the partial sciatic nerve that reported upregulation of CCL1 and CCL3 but not CCL5 (68), although other studies using different models do report upregulation.

Nerve injury upregulates IL-6 and reduction of IL-6 reduces neuropathic pain (65, 69, 70). It has been proposed that TNF α upregulates IL-6 expression *via* a NF- κ B pathway (71). Our results are not consistent with that since IL-6 was upregulated early in the root compression animals in the absence of change in TNF α .

Thermal hyperalgesia was similar for both nerve and root compressions with the exception of PN21 where latencies following nerve compression returned to baseline 7 days postsurgery but were lower than controls at PS14. Thermal latencies were significantly reduced at PS7 but returned to control levels at PS14. Mechanical allodynia likewise was similar between the root and nerve compression treatments, but the effects differed from those of the thermal hyperalgesia. There was a slight allodynia at PN10 for the nerve compression compared to naïve rats and for root compression, there was a difference at PS1 following root compression, but again only compared to the naïve animals. When nerve compression was performed at PN14, there was a small but persistent allodynia that lasted until PS14, the last day tested.

There were several differences between the results with the compression model and those with various nerve ligation models (see **Table 1**). First, thermal hyperalgesia was more prolonged in the PN28-day old surgical animals compared to the mechanical allodynia. In contrast, in adult compression studies, mechanical allodynia is persistent (50). In developmental studies, the delayed appearance of neuropathic pain is specific for a mechanical stimulus, not thermal stimuli (17, 21, 72). There are at least two possible reasons for this. The first is the nature and duration of the injury. The compression as used here is brief whereas in the ligation models the injury is chronic. Thus ligation is a more continuous injury compared to single acute compression. This may be particularly important in infant and young animals where sensory neurons from the DRG are still growing during the first

week of life (73–75) perhaps changing the nature of the ligation as the axons grown. Second, the root injury was just proximal to the DRG and the nerve injury just distal to the DRG. As found here, the changes in pain sensitivity were similar between the two injuries but the nerve injury induced fewer changes in immune markers sensitivity particularly at PN28. We know of no root ligation models during early development, but dorsal rhizotomy (76) or dorsal root constrictions (77) produce neuropathic pain in the adult. Although there are no developmental dorsal root constriction models, dorsal rhizotomy in infants does not induce self-mutilation whereas it does in 40% of juveniles and 80% of adults (78). Third, the prior infant injury models induced nerve injury in the hindpaw/lumbar spinal cord whereas the injury described here was forepaw/cervical spinal cord. We know of no data suggesting differences however. Although we did not quantify anatomical changes in the spinal cord here, future work should directly compare brief compression injury to comparable ligation models, including changes in dorsal horn structure and function to determine if those changes could account for differences among injury models.

We found no injury-induced changes in anti-inflammatory cytokines such as IL-4 and IL-10, despite the elegant data showing that these cytokines are developmentally regulated and actively suppress expression of proinflammatory cytokines/chemokines and neuropathic pain prior to 33 days of age in a spared nerve injury (SNI) model (48). We also found that these cytokines were overexpressed in the untreated infants relative to 42-day old rats. Both IL-4 and IL-10 were about three- to fourfold enriched at PN10–21 compared to adolescents. We did not measure protein levels of release of these cytokines. Thus, it is possible that expression of IL-4 and IL-10, although showing the normal reduction with age, did not reflect the changes following compression that were found in a ligation model (48), although others have reported greater expression of IL-4 in adults than infants using the SNI

method (17). Alternatively, the single compression injury, unlike the SNI, may not stimulate the dominant anti-inflammatory response induced in that model.

Finally, our data are consistent with the existent literature that early injury does not induced neuropathic pain, possibly because of less immune activation, and extend those data to a nerve and nerve root compression injury. In the current studies, we did not find evidence of a delayed mechanical allodynia as reported previously (21, 48, 72). Further work will be needed to understand these differences, identifying mechanisms that account for both the similar and different results and to extend them to other assays (e.g., cold allodynia) that also show onset of neuropathic pain after the third week of life in the rodent. Moreover, whether or not there are sex differences in the development of neuropathic pain at these ages is an important question given that there are sex differences in adults and that they are mediated by different components of the immune system [e.g., Ref. (79–82)].

AUTHOR CONTRIBUTIONS

GB and BW conceived and designed the study and wrote the manuscript. SW and CW adapted the surgical methods for infants and performed the surgery. SW tested the subjects.

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Lifetime Modulation of the Pain System *via* Neuroimmune and Neuroendocrine Interactions

Ihssane Zouikr^{1*} and Bianka Karshikoff^{2,3}

¹Laboratory for Molecular Mechanisms of Thalamus Development, RIKEN BSI, Wako, Japan, ²Department of Clinical Neuroscience, Division for Psychology, Karolinska Institutet, Solna, Sweden, ³Stress Research Institute, Stockholm University, Stockholm, Sweden

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Hans-Peter Hartung,
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*Correspondence:

Ihssane Zouikr
ihssane.zouikr@uon.edu.au

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Chronic pain is a debilitating condition that still is challenging both clinicians and researchers. Despite intense research, it is still not clear why some individuals develop chronic pain while others do not or how to heal this disease. In this review, we argue for a multisystem approach to understand chronic pain. Pain is not only to be viewed simply as a result of aberrant neuronal activity but also as a result of adverse early-life experiences that impact an individual's endocrine, immune, and nervous systems and changes which in turn program the pain system. First, we give an overview of the ontogeny of the central nervous system, endocrine, and immune systems and their windows of vulnerability. Thereafter, we summarize human and animal findings from our laboratories and others that point to an important role of the endocrine and immune systems in modulating pain sensitivity. Taking "early-life history" into account, together with the past and current immunological and endocrine status of chronic pain patients, is a necessary step to understand chronic pain pathophysiology and assist clinicians in tailoring the best therapeutic approach.

Keywords: psychoneuroimmunology, lipopolysaccharide, inflammation, pain, neuroimmunology, neuroendocrinology, hypothalamo-pituitary-adrenal axis, stress

INTRODUCTION

The pain system is modulated by neuroimmune and neuroendocrine mechanisms from embryonic development throughout life. Unlike the traditional reductionist view that posits that pain is solely due to aberrant spinal and supraspinal neuronal activity, we now understand pain in the context of a complex multisystem comprising well-organized interactions between neuroendocrine and neuroimmune systems (1). The changes in the nervous system induced by the immune system and the endocrine system are of both structural and functional character and are a part of the normal, adaptive development of the pain system. However, an adaptation that is advantageous in one situation may pose a risk factor in another. Exposure to a wide range of stressors, from physical injury (such as incision) to infection and inflammation [as induced by, e.g., lipopolysaccharide (LPS)], activates the hypothalamo-pituitary-adrenal (HPA) axis as well as peripheral and central immune responses and reorganizes the sensitivity of the pain system (2–5). The HPA axis and neuroimmune activation are of importance in determining long-term pathological states such as chronic pain.

Treating chronic pain is complicated by the wide individual differences in symptoms and treatment response. Chronic pain is also associated with a high incidence of psychiatric comorbidity

(6) and is often present with other primary diagnoses, such as inflammatory disease. Furthermore, stress is often directly targeted in behavioral treatment strategies for chronic pain (7), as part of an integrated treatment approach (8, 9). In this study, we explore some of the biological mechanisms that may form the foundations of the complexity seen in clinical pain.

This review focuses on some of the mechanisms involved in the maturation of the nervous system, which define the function of the pain system later in life. We highlight the importance of neuroimmune and neuroendocrine interactions very early in life in the programming of the pain system. We also discuss how the immune system and the endocrine system continue to modulate pain processing throughout life and about the significance of these interactions for chronic pain.

ONTOGENY OF THE CENTRAL NERVOUS SYSTEM (CNS) DURING THE PRENATAL AND POSTNATAL PERIOD

Neuronal circuits are forged by sensory experiences. Exposure to environmental stressors during a critical period of brain ontogeny, when neuronal circuits are particularly sensitive to modification by experience, can have long-term consequences on neural circuits, ultimately affecting behavior (10). Although our genetic makeup determines much of the structure and function of the nervous system, the environment where the individual is born, as well as the environmental conditions that will accompany the individual throughout his/her life, plays a crucial role in tailoring the neuronal properties. The postnatal developing nervous system responds to the external world to shape its neural circuits in order to subserve a particular function (i.e., vision, auditory, touch, etc.). In normal conditions (i.e., in the absence of any adverse events), non-stressful early experience specifies a neural trajectory to the best possible circuits of connectivity. In other words, non-efficient connections are eliminated and those that are functionally stable remain. However, if exposed to stress—whether it is of physical, physiological, psychological, or viral/bacterial nature—during a time when the brain is still undergoing fine-tuned maturation, the process of synaptic plasticity, or synaptic tuning can go seriously wrong, affecting the behavioral outcome.

Early Development of the Human Brain

During the prenatal period, the brain produces approximately 250,000 cells per minute (11). Neuronal migration occurs between gestational week (GW) 8 and 16 forming the subventricular zone (SVZ) (12). Around GW 16, neurons reach their final target and begin to form connections among brain regions (13). Synapse formation in both the auditory and prefrontal cortices begins around GW 27 (14). During the beginning of the third trimester, synaptogenesis occurs with a rate of approximately 40,000 synapses per minute (15). Subsequently, myelination as well as proliferation and differentiation of oligodendrocytes (cells that produce myelin) take place. After birth, the size of the brain continues to increase dramatically, with intense metabolic changes associated with synapse formation and axonal growth during the first 3 months of postnatal life (16). The way the complex human

brain develops and matures is through a significant increase in volume due to overproduction of synapses, myelination, and connections during infancy, followed by the elimination of less efficient synapses *via* pruning (17). Most importantly, the developmental trajectory of the neocortex is different depending on brain regions. For instance, the primary visual cortex undergoes significant maturation during the first 3 months of life, whereas the primary auditory cortex continues to mature over the first 3 years of life (18). The bilateral thalamic connectivity to the prefrontal cortex (PFC) is increased gradually from childhood to late teens (19), and synaptic pruning in the PFC continues to occur in mid-adolescence (14). The relatively late maturation of thalamo-PFC synaptic connections implies that key connections involved in complex cognitive functions, including pain, are still undergoing fine-tuned maturation in early postnatal life. Consequently, exposure to stressful events such as viral/bacterial infections during postnatal life is likely to be able to alter key neural circuits involved in pain processing. This may lead to altered pain responses later in life. At present, there is a paucity of research tackling this question, and further studies investigating the impact of early-life stress on neural circuits involved in pain processing are needed.

What Animal Models Reveal about Neurogenesis and Synaptic Plasticity

The traditional dogma posits that the postnatal brain (including adult brain) possesses a fixed number of neurons that are generated from birth and that no neurogenesis or synaptic plasticity is possible in the adult brain (20). However, it is now clear that neurogenesis and synaptic plasticity continue to occur in the adult brain, although at a lower rate. Findings from studies that used standard neuronal markers, such as NeuN and bromodeoxyuridine (BrdU), have detected postnatal neurogenesis both in primates and rodents. NeuN⁺/BrdU⁺ cells were detected particularly in two regions: the SVZ–olfactory bulb and the subgranular zone (SGZ)–hippocampal granule cell layer (21–25). Regarding synaptic plasticity in the adult brain, pioneer studies by Merzenich et al. demonstrated that amputation of one finger in adult monkeys resulted in deafference of the devoted territory within the somatosensory cortex and that this region compensated by receiving inputs from neighboring fingers (26). Later on, Robertson and Irvine showed that similar compensatory mechanisms and cortical rearrangement occurred in the auditory cortex following lesion of the cochlea (27).

In rats, PFC neural circuits undergo significant changes during the perinatal period. The myelination of the medial PFC (mPFC) is very low at P7, increases gradually over the period P21–P50, and reaches peak level at P90 (28). The ontogenic development of the PFC implies that this region, which plays a critical role in cognitive functioning and pain processing (29), is particularly susceptible to environmental stimuli during the neonatal period. Consequently, exposure to stressful events during this period is likely to alter the neural circuits within the PFC—and consequently pain processing later in life. Indeed, sensory, painful, or stressful experience has been shown to change the dendritic and spine morphology in this area. A combination of prenatal

stress (E14–E21) and maternal separation (P2–P21) resulted in increased c-Fos expression in the mPFC and reduced dendritic length and dendritic spines of mPFC neurons (30). A recent study has found that pyramidal neurons from the mPFC of spared nerve injury (SNI) rats are characterized by longer basal dendrites and increased spine density compared to sham-operated animals (29). Electrophysiological recording of mPFC pyramidal neurons from SNI rats revealed increased NMDA/AMPA ratio in currents evoked by stimulation of layer 5 (29). However, convincing data linking directly altered PFC neural circuits following early-life stress to future pain responses are still lacking.

Taken together, these data suggest that the perinatal, up to and including the early childhood period, is a time of high plasticity for the brain and adverse events occurring during this critical period of cellular proliferation, differentiation, and maturation can interfere with the normal developmental trajectory of the brain, resulting in structural and/or functional changes in cells, tissues, or organ systems. These changes are proposed to potentially lead to increased susceptibility to neurodevelopmental disorders in later life (31–33) and may also be critical for determining adult pain responses and potentially the susceptibility to develop chronic pain.

Early-Life Development of the Pain System

One of the neuronal systems that undergo significant malleability during the perinatal period is the nociceptive system. For instance, at embryonic day (E) 15–17 myelinated A fibers are the first to penetrate the spinal lumbar cord before the subsequent projection of C fibers into the substantia gelatinosa (lamina II, superficial dorsal horn that contains nociceptive-specific neurons) at E19 (34). During the neonatal period, lamina II is innervated by both A- and C fibers. During the first 3 weeks of postnatal age, a withdrawal of A fiber primary afferents into deeper laminae is noticed, and C fibers exclusively innervate lamina II at the adult stage (35). This developmental pattern of nociceptive fibers is of particular relevance to the concept that early-life insults are able to alter the neuroanatomical components of nociception (including nociceptive fibers), leading to altered pain responses later in life. For instance, skin wound during the neonatal period is associated with hyperinnervation of the wounded area by both A δ and C fibers (36, 37). This hyperinnervation of nociceptive fibers can lead to peripheral sensitization and increased pain sensitivity (i.e., hyperalgesia). Despite the apparent lack of maturity of the nociceptive system, overall, younger animals are markedly more sensitive to noxious stimuli than their adult counterparts (38). Their behavioral output may, however, differ from adult animals. The withdrawal threshold from heat stimuli is lower in young animals compared to adults, and neonatal rats are significantly more (i.e., 10-fold higher) sensitive to formalin injection than preadolescent rats who require higher formalin doses to elicit the formalin-induced behavioral responses (38). For example, until P10, injection of formalin into the hind paw elicits predominantly non-specific whole body movement (i.e., jerking), whereas the formalin-induced specific behaviors such as hind paw shaking, flexion, and licking appears only after P10 (39). Of particular

interest, recent studies predominantly from Hathway et al. elegantly demonstrated that the descending inhibitory control of spinal nociceptive reflexes from the periaqueductal gray (PAG) to rostroventral medullary (RVM) in rats undergoes an important developmental switch from facilitatory in young rats to inhibitory in adult rats (40–42). This developmental switch was found to be driven by opioid actions on RVM, as microinjection of the μ -opioid agonist [D-Ala², N-MePhe⁴, glycol]-enkephalin (DAMGO) into RVM facilitates spinal nociceptive reflexes in preadolescent rats (P21), but elicited antinociceptive actions in adult rats (42), and similar response pattern has also been recently shown to occur at the PAG level (41).

Overall, a number of neural systems, including those involved in pain modulation, are characterized by significant malleability illustrated by major structural and functional rearrangements in neural circuits following insult. This injury-induced plasticity renders the nociceptive system more vulnerable to future challenges. Why certain patients develop chronic pain while others do not might in fact result from different early-life experiences in these patients, which may have programmed the pain system differently later in life. Therefore, taking “early-life history” into account is a necessary step to understand chronic pain pathophysiology and developing individual-based therapeutic strategies (43).

ONTOGENY OF THE HPA AXIS DURING THE PERINATAL PERIOD

Prenatal Development

The experience of stress, from an evolutionary perspective, is very important in promoting survival of an organism. A fundamental system that is subjected to programming by early-life events is the neuroendocrine axis that mediates the stress response, the HPA axis (44, 45). Activation of this system starts with the recruitment of neurons within the paraventricular nucleus of the hypothalamus (PVN), and the end product is the release from the adrenal cortex of corticosterone for rodents or cortisol for humans, *via* the release of corticotropin-releasing hormone (CRH) and adrenocorticotrophic hormone (ACTH) [the HPA axis has been extensively reviewed elsewhere, please see Ref. (46)]. During pregnancy, there is an increase in CRH production in the placenta and fetal membranes. The gradual increase in maternal HPA axis activity during this period leads to maternal hypercortisolemia (47, 48). The fetus has much lower levels of glucocorticoids than its mother although endogenous glucocorticoids can cross the placenta easily. A total of 10–20% of cortisol present in the amniotic liquid is from maternal origin, while the remaining 80–90% gets converted into inactive cortisone by an enzyme, 11 β -HSD2, to protect the fetus's brain from excess glucocorticoids, which can be neurotoxic (49). During the third trimester, fetal 11 β -HSD2 levels decrease, and the fetus is exposed to high levels of CRH and cortisol. This rise in CRH and cortisol levels is thought to play an important role in the maturation of organs and preparation of the fetus to the *ex utero* environment (50). The hippocampus plays a key role in regulating homeostatic levels of glucocorticoids under conditions of stress, and CRF has been shown to modulate the

electrical activity of hippocampal neurons (51). Glucocorticoid receptor (GR) mRNAs were detected in human fetal hippocampus at 24 GWs (52). Additionally, fibers expressing CRH have been detected in humans by GW16 (53), and the release of CRH into the pituitary has been reported to occur at GW11.5 (54). At the pituitary, a basic adenohypophysis can be detected at GW6 (55), and by GW8 the pituitary reaches mature stage and can release ACTH (56).

In rodents, GR mRNA can be detected in the telencephalon as early as E12.5 with high expression seen in the anterior hypothalamus, pons, spinal cord, and pituitary gland (57). At E14.5, the expression of GR mRNA significantly increases in the ventral spinal cord and the thalamus and undergoes a moderate decrease in these regions by E15.5. An increase in GR mRNA levels is observed at the same time point in other regions including the neocortex, cerebellum, and basal ganglia (57). In the PVN, GR mRNA can be visible at E16, although it is not clear whether this PVN GR is functional at this stage (58). During the late gestation (E17–E19), GR mRNA is localized in the hippocampus, thalamus, and the amygdala (58). Mineralocorticoid receptors (MR) ontogenetic expression, however, follows a different pattern in rodents. MR mRNA expression cannot be detected before E15.5 when a moderate expression is observed in pituitary gland, brain stem, tegmentum, and neuroepithelium of the septum and pallidum (57). MR mRNA expression is first seen in the hypothalamus at E17.5 and by E19.5 there is a dramatic increase in MR mRNA expression in the hippocampus, septum, anterior hypothalamus, PAG area, and brainstem neuroepithelium (57). Regarding the ontogeny of 11 β -HSD2 mRNA (encoding an enzyme that converts corticosterone into its inactive form) in rodents, the expression of 11 β -HSD2 mRNA is observed at E11.5 on hippocampal and subicular regions, neocortex, septum, and posterior hypothalamic area. At E14.5, the expression intensity of 11 β -HSD2 mRNA starts to decline in the neocortex, pallidal area, and spinal cord, and by E15.5 11 β -HSD2 mRNA is restricted to the thalamus, midbrain, striatum, cerebellum, hypothalamus, medulla, and pallidum (57).

Postnatal Modulation

There is a particular period called “the stress hyporesponsive period” (SHRP) from P4 to P14 in rats and from P2 to P12 in mice during which corticosterone levels, as well as ACTH, are maintained at low levels even in the presence of mild stress (59). Although, it is generally accepted that pups do not respond to stress with an elevated HPA axis activity during the SHRP period, it has been reported that 12 day-old pups that were separated from their mothers for 24 h with no access to food or water showed a significant increase in both basal and stress-induced corticosterone and ACTH secretion (59, 60). These results indicate that the HPA axis is particularly sensitive to maternal care even during the SHRP. During this period, high expression of CRH is observed in the PVN, whereas hippocampal GR expression is low at birth and increases gradually during the SHRP (61). *In situ* hybridization studies in marmoset showed that the ontogenetic profile of MR and GR is different during the postnatal period. Although GR mRNA expression in the dentate gyrus is higher in 4–6 week-old marmoset than in neonates (P1–P2), juveniles

(4–5 months), and adult (3–6 years), MR mRNA expression was developmentally consistent in the hippocampus and PVN throughout life (62).

Although we need to proceed with caution when extrapolating from animal studies to humans, the development of the brain in terms of synapse formation and brain growth rate in a P6 rat is relatively equivalent to 38–40 weeks of gestation in humans (63, 64). For obvious ethical and methodological reasons, human data regarding the ontogenetic development of HPA axis are lacking. However, we can conclude from the abovementioned animal data that the prenatal period together with the first 2 weeks of postnatal life constitute a window of significant plasticity for the neuroendocrine system. Homeostasis of the neuroendocrine function, and consequently any physiological system that is under the influence of this system (e.g., pain), is needed for normal neuroendocrine development. Excessive stress that may challenge or perturb the neuroendocrine system when it is still developing could potentially have far-reaching consequences. This way, early-life stress may alter pain, neuroimmune, and neuroendocrine responses for life (4, 65–69).

EARLY DEVELOPMENT OF THE IMMUNE SYSTEM

Immaturity of the Neonatal Immune System and Susceptibility to Infection

Infant mortality due to infection is high particularly in developing countries with a high prevalence of infection during the neonatal period (70). This high susceptibility of neonates and preterm infants to infection is thought to be due to immaturity of the neonatal immune system. Analysis of umbilical cord from preterm infants revealed fewer naïve CD8⁺ T cells and regulatory CD31 expression compared to full-term neonates (71). T cells play an important role in the control of intracellular infections. Both human and murine neonates lack mucosally distributed memory CD8⁺ T cells. Although T cell and cytokine mRNA levels [i.e., interleukin (IL)-1 β , IL-6, and IFN- δ] can be detected in the thymus of mice from GD15 (72), neonatal mouse macrophages do not react in an adequate way early in life. For example, T-cells are characterized by lower IFN- δ responses following stimulation (73, 74). *Ex vivo* stimulation with the bacterial mimetic LPS in mice produced much less pro-inflammatory and anti-inflammatory cytokines response in neonates compared to adult mice (75). The same trend was observed in a human study whereby neonatal monocytes and dendritic cells produced less tumor necrosis factor (TNF)-alpha, IL-12, and IL-6 following LPS stimulation (76). When stimulated with an anti-CD3 antibody, neonatal T cell proliferation significantly decreased compared to adult T cell proliferation. This attenuation of proliferation in neonatal T cells was restored to adult levels following the addition of exogenous IL-2 (77). Furthermore, the total cell number of T cell subtypes (CD4⁺, CD8⁺, and Thy1⁺) is markedly lower in the spleen and lymphoid nodes in P4 mice compared to adult mice (78). Similarly, the function of antigen-presenting cells (APCs) is markedly decreased in human and murine neonates compared to adults (78). Treatment of both immunocompetent

and immunodeficient mice with IL-12, a cytokine produced by APCs (79), prior to inoculation with the parasite *Cryptosporidium parvum* oocysts markedly reduced the severity of infection (80). Additionally, neonatal mice exhibited reduced levels of peripheral IL-12, and mice treated with IL-12 24 h after birth displayed increased levels of IFN- δ and IL-10 mRNA in the spleen (81). Adult humans exhibited much higher levels of granzyme B⁺ effector differentiated memory CD8⁺ T cells, which are thought to be the first responders to infections (82), than human neonates (83).

The incidence of sepsis, defined as a systemic inflammatory condition that occurs following exposure to pathogenic microorganisms or their toxins, is more than 25 times higher in infants less than 1 year compared to children from 1 to 14 years of age and constitutes a major risk of mortality and morbidity in the pediatric population (84). The incidence of infections is particularly high during the first postnatal weeks and rapidly decreases thereafter (85). Common causes of infections in neonates include commensal bacteria such as *Escherichia coli* (85). Both adaptive and innate neonatal immune responses are relatively immature as indicated by a lack of preexisting memory and decreased Th1-type responses (86, 87) as well as impaired production of TNF α following exposure to LPS (88, 89). Neonatal monocyte dendritic cells (moDC) also showed decreased production of interferon- β (IFN- β) in response to *in vitro* stimulation with LPS compared to mature adult moDC (89, 90). Additionally, whole blood neutrophil concentrations in 1-month children are shown to be lower than those in adults (91).

This immaturity of the immune system during neonatal life may thus predispose the neonatal immune system to infection, both of intra- and extracellular types. Overall, bacterial infection is considered the number one cause of perinatal infection in newborns worldwide (92, 93), which results in increased infant mortality particularly in developing countries (92, 94). In the coming sections, we argue that this sensitivity of the immune system early in life may have long-lasting effects in the adult organism.

Infection As a Perinatal Stressor

Exposure to pathogens early in life is a common event and is considered to play a crucial role in priming the neuroendocrine–neuroimmune interface (95). An infection may not only be life-threatening to an infant but may also reorganize the function of the nervous system, due to the tight interplay between the nervous and immune systems. Human and animal studies have demonstrated that perinatal exposure to an immune challenge can produce changes in the CNS structure and function, leading to an increased risk of developing behavioral and psychopathological alterations later in life (66, 96–100). For instance, offspring from mothers exposed to infections such as influenza, LPS, and viral RNA (Poly I:C) during pregnancy have higher risk of developing schizophrenia and autism (101–106). A significant number of human and animal studies have also indicated that perinatal infection can alter immune (97, 107–110), metabolic (111, 112), reproductive (113, 114), endocrine (95, 115, 116), neurological (117, 118), and cognitive and behavioral responses later in life (98, 119, 120). Interestingly, exposure to LPS in rodents and humans can also cause pain facilitation such as thermal hyperalgesia, mechanical allodynia, and hyperalgesia (121–125). Such

behavioral findings appear to be the result of altered peripheral and central cytokine activity (122, 126–128). Increased levels of pro-inflammatory cytokines, including IL-1 β , TNF- α , and IL-6 produced by the maternal or fetal immune system, have been linked to abnormal brain development and increased risk of developing psychopathology (96, 98–100). Moreover, higher amounts of IL-6 in the amniotic fluid following bacterial infection during pregnancy have been previously reported to strongly correlate with increased mortality rates and brain injury (129).

Taken together, these findings highlight the fundamental role of the microbial environment in programming behavioral and neural responses. In order to understand the mechanisms of perinatal neuroendocrine–neuroimmune interaction, researchers employ experimental models that mimic the antigenic actions of infection.

LPS AS AN EXPERIMENTAL IMMUNOLOGICAL STRESSOR

Lipopolysaccharide, a complex glycolipid that is the major component of Gram-negative cell wall usually derived from *Salmonella enteritidis* or *E. coli*, is a powerful activator of innate immune responses and induces behavioral symptomatology in the host largely identical to those induced by live bacterial infection (130, 131). LPS-induced inflammation model presents well-known advantages, the primary one being that LPS does not replicate, allowing tight control of dosage and limiting the confounding nature of infection as compared to live bacteria models. LPS is commonly used to understand the complexities of the neuroimmune–neuroendocrine relationship and has been demonstrated to be a reliable activator of innate immune responses (97, 108) and HPA axis (66, 95, 108, 116, 132). Thus, LPS acts as an experimental systemic immunological stressor (133).

Lipopolysaccharide activates toll-like receptors and initiates a cascade of signalization leading to cytokine production that is crucial for infection clearance (134). Monocytes, neutrophils, macrophages, dendritic cells, and mast cells all express TLR4 at their surface membrane (135–137). Upon activation of the TLR4/MD2 complex by LPS, a series of phosphorylation steps are activated, leading to the phosphorylation of inhibitory (I) κ B, which releases nuclear factor (NF)- κ B from its complex (138). NF- κ B is subsequently translocated into the nucleus where it activates the transcription of pro-inflammatory cytokines such as IL-1 β , TNF α , and IL-6, as well as anti-inflammatory cytokines such as IL-1 receptor antagonist (IL-1ra) and IL-10 (139, 140). Cytokines released in the blood stream are able to activate the release of cyclooxygenase (COX)-2 from the hypothalamus to induce hyperalgesia (141). COX-2 also stimulates the conversion of arachidonic acid into prostaglandins (PGE₂), which acts in the vascular organ of the lamina terminalis and in the ventromedial preoptic area of the anterior hypothalamus to stimulate heat conservation *via* cutaneous vasoconstriction and attenuation of sweating, and heat production *via* increases in the metabolism of brown adipose tissue (142). Circulating IL-1 β is also known to directly activate hypothalamic PVN to stimulate the release of corticosterone from adrenal cortex (143, 144). LPS activation of Kupffer cells in the liver is also known to activate the release

of IL-1 β that can contribute to hyperalgesia *via* vagal afferences (145), as vagotomy abolishes the LPS-induced hyperalgesia (145).

Neonatal LPS Exposure Changes Immune Responses Later in Life

Several lines of evidence from clinical and animal work suggest that exposure to LPS during the neonatal period is associated with altered immune responses later in life (66, 97, 109, 146–150). Most importantly, long-term inflammatory responses within the CNS are greatly influenced by immunological stressors early in life. Incubation of cord blood from 1-month old children with LPS for 5 h resulted in increased mRNA expression of IL-6 and TNF α compared to cord blood from the same age incubated with medium (146). In rats, neonatal LPS exposure produces immediate upregulation of gene expression of chemokines and cytokines within the neonatal brain, as indicated by upregulation of mRNA levels of Ccl7, Cxcl1, Cxcl10, IL-1 β , and IL-6 in the hippocampus 2 h following LPS exposure in rat pups at PND 4 (151). The effect of neonatal LPS exposure on cytokine levels in limbic areas can persist into adulthood. Our laboratory has previously shown that neonatal LPS exposure at PNDs 3 and 5 results in increased IL-1 β and TNF α protein levels in the hippocampus following exposure to restraint stress in adulthood (66). Recent investigations point toward a critical role played by the hippocampus in modulating pain *via* upregulation of IL-1 β expression (152). del Rey et al. documented a strong correlation between increased hippocampal IL-1 β transcripts and mechanical allodynia in chronic constriction injury and spared nerve injury (SNI) models (152). However, it is not known whether changes in protein levels of IL-1 β in the hippocampus contribute to increased pain sensitivity in inflammatory pain models (i.e., formalin test). Neonatal immune challenge has also been reported to alter febrile responses later in life (147, 148, 150). Fever is considered an important component of the innate immune response and is thought to play a crucial role in survival through its ability to efficiently clear the pathogen while limiting the extent of inflammatory damage (153, 154). Animals prevented from developing fever have higher risk of morbidity and mortality than animals that are allowed to develop fever (155). Rats exposed to LPS at P14 exhibited attenuated fever responses following a subsequent LPS challenge (147, 149) or stress (150) in adulthood. The effect of neonatal LPS exposure on adult febrile responses is thought to be mediated by pro-inflammatory cytokines, as neonatally LPS-treated rats displayed significantly reduced plasma levels of TNF α and IL-6 following subsequent LPS exposure in adulthood. This reduction in turn was strongly correlated with the observed attenuated febrile responses in LPS animals (147). Interestingly, basal maintenance of body temperature in adult rats was not affected by neonatal LPS administration (110). This finding implies that a single LPS exposure is not able to alter febrile responses later in life, but that a “second hit” is necessary to “unmask” the altered febrile responses following a neonatal immune challenge. Central levels of PGE₂ and specifically in the preoptic region, a region involved in the febrigenic thermoeffector pathways (156, 157), have also been targeted as potential mechanisms mediating the attenuated febrile responses following a neonatal immune challenge. For

instance, PGE₂ levels in the preoptic area were increased in rats exposed to LPS at P14 (150). Additionally, glucocorticoids play a critical role in inducing the febrile response, as adrenalectomy or blockade of GRs using the GR antagonist RU-486 abolished the fever induced by neonatal exposure to LPS (147). Finally, our laboratory has previously demonstrated that rats exposed to LPS at PNDs 3 and 5 displayed increased susceptibility to tumor and lung metastases following exposure to stress in adulthood (97, 108). Moreover, neonatal immune challenge produced reduced NK cell activity and increased neuroendocrine responsivity to restraint stress in adulthood (97, 108).

Taken together, an early immunological stressor has profound effects on the immunological reaction pattern later in life, leading to altered neuroimmune function at subsequent exposures to immunological challenges. This implies that what the immune system of an organism has been exposed to very early in life will in fact define its capacity to defeat pathogens later in life.

Impact of Neonatal LPS Exposure on Endocrine Function

Microbial microbiota can affect the postnatal development of HPA axis, and an increasing body of evidence has demonstrated that neonatal exposure to LPS is associated with long-term alterations in HPA axis activity (66, 97, 116, 149, 158). Neonatal exposure to LPS during P3 and 5 has been reported to increase circulating levels of corticosterone at both time points (66, 132, 159), suggesting that neonatal LPS exposure is capable of altering HPA axis function during the SHRP. This alteration in HPA axis function following a neonatal immune challenge persists throughout the life of the animal. Adult rats treated with LPS as neonates displayed enhanced plasma corticosterone and ACTH levels in response to restraint stress, noise stress, or in response to a second LPS hit in adulthood (66, 95, 97, 116, 132). This altered peripheral endocrine response was also accompanied by central neuroendocrine changes, as indicated by increased CRH mRNA levels in the PVN and decreased GR density in the hypothalamus, hippocampus, and frontal cortex following exposure to stress in adulthood (95). These structures are known to mediate the inhibitory effects of glucocorticoids on CRH synthesis in the PVN and the release of ACTH following stress (160, 161), suggesting a decreased negative feedback sensitivity to glucocorticoids and, thus, an enhanced HPA responsiveness to stress following a neonatal immune challenge. We have demonstrated in our laboratory that dual exposure to LPS during P3 and P5 in rats is associated with increased circulating corticosterone at P7 and P22, but not P13, 1 h following injection of formalin into the hind paw (68). P22 rats neonatally treated with LPS also exhibited a trend toward decreased GR mRNA in the hypothalamus (68).

Overall, these data suggest that exposure to LPS during the neonatal period can reprogram the neuroendocrine axis. This reprogramming increases the reactivity of animals to a second physiological challenge later in life. Pain is an aversive experience and, therefore, capable of activating the HPA axis (162). Given that neonatal LPS exposure has been associated with increased release of peripheral and central pro-inflammatory cytokines later in life (66, 151) and considering the well-established role of

pro-inflammatory cytokines in producing hyperalgesia (145), it is reasonable to assume that neonatal LPS exposure is likely to be associated with increased pain sensitivity later in life.

Impact of Neonatal Exposure to LPS on Nociceptive Responses

The first postnatal week (P7–P10) of rodent's life is equivalent to the last trimester in humans (36–40 GW) in terms of brain growth, gliogenesis, axonal and dendritic density, as well as consolidation of the immune system (11, 163–165). Preterm infants are, as discussed earlier, at high risk of infection during the neonatal period. Early-life infections in turn are known to be the cause of attenuated neurodevelopmental outcomes in these vulnerable infants (166). It is, therefore, important to address the impact of immune challenge on pain sensitivity later in life. Boisse et al found that administration of LPS at P14 in rats produced thermal and mechanical hyperalgesia that paralleled the enhanced expression of COX-2 protein levels in the lumbar spinal cord (141). Although this study did not directly demonstrate that the increased level of COX in the spinal cord contributed to the observed hyperalgesia in LPS-treated animals, it suggested a potential role of prostaglandins in mediating the LPS-induced hyperalgesia. Increased COX mRNA levels were also observed 4 h following LPS injection in P3 and P21 rats (P0 is birth) (167). A number of studies from our laboratory have indicated that dual exposure of LPS during P3 and 5 in rats produced long-term alterations in inflammatory pain responses later in life. Neonatal LPS administration evoked increased formalin-induced behavioral responses (i.e., flinching and licking) in P13, 22, and adult rats (4, 68, 168). The LPS-induced hyperalgesia observed in P22 rats coincided with altered HPA axis activity, as indicated by increased circulating corticosterone and decreased GR hypothalamic mRNA 1 h postformalin injection, as well as altered immune responses following formalin injection as indicated by increased mast cell degranulation and increased circulating IL-1 β (4, 68). Moreover, the LPS-induced hyperalgesia in pre-adolescent rats was accompanied by altered spinal dorsal horn (SDH) intrinsic properties, as well as decreased neuronal activity (i.e., Fos expression) in the PAG (68, 168). LPS-treated adult rats exhibited hyperalgesia that coincided with central neuroimmune changes, as indicated by increased IL-1 β in the hippocampus 1 h postformalin injection. No differences were observed in peripheral IL-1 β release or mast cell degranulation (4). Although we reported enhanced hippocampal IL β in LPS-treated adult rats, we do not know which immune cell releases this pro-inflammatory cytokine following neonatal immune challenge and subsequent inflammatory challenge. Of particular interest, hippocampal parenchyma astrocytes have been recently shown to produce the cytokine CCL2 24 h post-LPS injection in adult mice (169), suggesting an important role of astrocytes in the neuroinflammation produced by systemic LPS injection.

Taken together, these data challenge the traditional concept that pain is originating solely from activation of neurons and suggest that components of the immune system play an imminent role in modulating pain sensitivity. Using LPS as a model of infection, LPS-induced hyperalgesia arises by both peripheral and central

mechanisms. Peripherally, LPS triggers, e.g., macrophages to release pro-inflammatory cytokines that sensitize nociceptors (145, 170, 171). In fact, LPS can directly activate TRPA1-expressing neurons independent of TLR4 (172). Centrally, LPS can activate microglial cells in the spinal cord and astrocytes in brain regions such as the hippocampus and produce hyperalgesia (169, 173).

THE NEUROIMMUNE INTERFACE IN PAIN IN THE ADULT ORGANISM

As discussed so far, the exposure to immunological stressors very early in development of an individual has far-reaching effects on neural structure and function as well as on the immune and HPA axis activity. We have also pointed to defining changes for the adult pain system. In the mature body, the systems are fully developed and less malleable. However, the immune system continues to affect the function of the nervous system in a manner that drives pain sensitivity, by inducing functional changes. In this section, we describe some acute neuroimmune interactions in pain perception. Such neuroimmune interaction may potentially be of importance for the transition from acute to chronic pain in a long-term perspective.

Animal Studies Demonstrate Inflammation-Induced Pain Sensitivity

The role of the immune system was traditionally viewed as protecting the organism from invading pathogens. However, it is now well established that the bidirectional interaction between the immune and nervous systems plays a crucial role in pain modulation (125, 174–177). Pro-inflammatory cytokines play an important role in this immune to brain bidirectional interaction (121, 145). When exposed to LPS, immune cells such as macrophages, monocytes, and mast cells release many pro-inflammatory cytokines such as IL-1 β , TNF- α , and IL-6 into the circulation creating an “inflammatory soup” condition that enhances pain sensitivity by sensitizing nociceptors (178–180). These pro-inflammatory cytokines also signal to the brain to induce a set of physiological responses including fever, lethargy, decreased social interaction, decreased sexual activity, and decreased food and water intake, increased circulating corticosterone, collectively known as sickness behavior (181–183). Importantly, pain facilitation or hyperalgesia is considered to be an integral part of sickness behavior (121, 125). Peripheral inflammation can lead to central neuroinflammation *via* many different ways. First, through vagal afferences since sub-diaphragmatic vagotomy reversed the hyperalgesia induced by IL-1 β or LPS (145). Alternatively, cytokines can access the brain through areas that lack the blood–brain barrier (BBB) such as the *organum vasculosum lamina terminalis* (184). LPS produces IL-1 β in the brain, which is initially restricted to choroid plexus and circumventricular organs, then diffuse to the brain side of BBB (185). Cytokines have also been suggested to enter the brain *via* active transport systems across the BBB (186, 187).

The first report on the impact of LPS exposure on pain responses was the study by Mason, who demonstrated that i.p. administration of LPS in adult rats significantly decreased tail flick latency, an effect that peaked at 1 h post-LPS administration

(123). The LPS-induced thermal hyperalgesia observed in adult rats was reversed following the administration of IL-1ra (188), indicating that IL-1 β is an important mediator of this hyperalgesia. Pro-inflammatory cytokines released by immune cells are known to induce hyperalgesia when administered both peripherally and centrally, particularly IL-1 β (145, 170, 189, 190). For instance, intracerebroventricular (ICV) administration of the recombinant human IL-1 β (rhIL-1 β) in rats induced thermal hyperalgesia (170), while ICV injection of the IL-1 β antagonist IL-1ra abolished this hyperalgesia (170). Intraplantar injection of IL-1 β has been associated with increased discharge of SDH neurons in response to non-noxious stimuli (190). Local administration of IL-1ra decreased the LPS-induced hyperalgesia (171).

Interleukin-1 β is also known to contribute to flinching responses in the formalin test given that an intraplantar injection in rats of antisera anti-IL-1 β prior to formalin injection significantly attenuated flinching responses in the formalin test (191). We have previously shown that rats exposed to LPS during the neonatal period displayed increased circulating IL-1 β at P22 in response to formalin injection (4). Adult rats previously subjected to neonatal immune challenge also displayed enhanced hippocampal IL-1 β that coincides with the LPS-induced hyperalgesia at this age (4). The source of this hippocampal IL-1 β is not known, but it is highly probable that it is originating from astrocytes or microglial cells within the hippocampus. Interestingly, at the same age (i.e., PND 22), and at the same time point following formalin injection (i.e., 1 h postformalin injection), we observed altered intrinsic properties of SDH, lamina I, and lamina II neurons in LPS-treated rats as indicated by lower input resistance compared to saline-treated rats (68).

Spinal dorsal horn neurons are the first component of the CNS to receive incoming noxious sensory information, and their output is determined by a combination of their synaptic inputs and intrinsic neuronal properties (192). Formalin injection is known to activate peripheral nerves, which results in turn in activation of dorsal horn neurons (193–195). Hind paw injection of formalin is associated with the release of numerous substances in the spinal cord, including prostaglandin E₂ (196). Bath application of prostaglandin E₂ results in changes in intrinsic properties of dorsal horn neurons including decreased input resistance (197). Since this change was only observed in LPS-treated preadolescent rats, it is possible that the neonatal exposure to LPS resulted in either an increase in pro-inflammatory cytokines within the spinal cord or an increased susceptibility of SDH neurons to pro-inflammatory cytokines. This assumption is confirmed by the fact that intrathecal administration of IL-1ra has been reported to block formalin-induced hyperalgesia (198). The source of spinal hyperalgesia seems to involve microglia and astrocytes since intrathecal administration of fluorocitrate, an inhibitor of glial metabolic function, blocked the formalin-induced hyperalgesia (198).

Additionally, IL-1 β has been documented to act supraspinally to induce hyperalgesia. For instance, microinjection of IL-1 β into the preoptic area of the hypothalamus is sufficient to induce thermal hyperalgesia (199). Of particular interest is the observation that IP or ICV administration of IL-1 β has been documented to produce an increase in plasma levels of corticosterone and ACTH,

an action that is mediated by the release of CRH from the PVN (144, 200). The neonatal immune challenge is likely to influence the generation of new neurons in the hippocampus. This assumption is confirmed by the fact that an intraplantar injection of the nociceptive inflammatory agent Complete Freund's Adjuvant at P8 results in more BrdU and doublecortin-labeled cells, both measures of newborn neurons, in the SGZ of the dentate gyrus (201). Whether such neurons release IL-1 β in response to neonatal LPS exposure remains to be determined.

At the peripheral level, the enhanced IL-1 β plasma levels observed at PND 22 in LPS-treated rats coincide with higher degree of mast cell degranulation, which was also accompanied by increased formalin-induced nociception (4). Mast cells are located in the vicinity of primary nociceptive neurons and vasculature and their degranulation has been reported to regulate the excitability of nociceptive nerve endings (202). Mast cell degranulation can also produce thermal hyperalgesia *via* the production of nerve growth factor (203). Previous studies have documented an important role of mast cells in formalin-induced nociception. Blocking mast cell activity using the mast cell stabilizer cromolyn abolished formalin-induced pain responses in the late phase (204). Interestingly, mast cells are also known to express receptor for IL-1 β and to produce IL-1 β following inflammation (205).

Inflammation-Induced Pain Sensitivity in Humans

The human physiology is much more sensitive to LPS provocation than that of rodents. To avoid the risk of sepsis, very low doses of LPS are used in humans (usually 0.2–4.0 ng/kg), the highest doses often requiring additional antipyretic pharmacological treatment. The most common dose for psychological research is around 0.4–1 ng/kg LPS from *E. coli*, which induces a clear rise of pro-inflammatory cytokines TNF α , IL-1 β , IL-6, and IL-8 in the blood (206–208). Human studies can also benefit from vaccinations of healthy individuals as an inflammatory model, and patients undergoing immunotherapy can be studied. The behavioral outcomes of experimental immune activation are very similar to sickness behavior exhibited by experimental animals; individuals report increased anxiety, worsened mood, and increased pain sensitivity (205, 209, 210). Appetite is reduced, and fatigue and anhedonia increase parallel to decreased social interest (126). The immune activation also disrupts memory and cognition and changes motivation (6, 211, 212). In human studies with the lowest LPS doses, the effects can in fact be so subtle that blinding can be maintained.

Pain Sensitivity during Immune Provocation

So far, only LPS stimulations have been used to study the pain system specifically in humans, and several studies have shown that experimental immune activation increases pain sensitivity in humans, too. Deep (muscular and visceral) pain is more readily affected than superficial (cutaneous and mechanical) pain (207, 213, 214). Also, the change in pain sensitivity usually correlates with peripheral cytokine levels. As in all experimental pain research, the mode of pain stimulation as well as the pain intensity applied may affect the outcome. Threshold pain is not processed exactly the same way as suprathreshold (intense)

pain, and pain from within the body is relayed to the brain in pathways partly distinct from those used to relay cutaneous pain (215). Also, the nociceptive effect may depend on the immunological pressure, i.e., the LPS dose in experimental models. Two studies show that threshold pressure pain sensitivity is affected the same way in men and women, despite the generally higher cytokine levels found in women during LPS stimulation (207, 216). Interestingly, no sex differences in psychological outcomes, such as anxiety or perceived health, are seen either despite the sex differences in cytokine release (207, 208). One study has, however, shown that women are indeed more affected by inflammation with regard to pain perception (207). In this study, the descending pain inhibition of women was weakened during LPS stimulation, while men remained unaffected. In parallel, women were more pain sensitive to intense cutaneous pain, too, while men only changed their perception of deep pain. Furthermore, one study using a high LPS dose (2.0 ng/kg) has in fact shown increased pain sensitivity to intense cutaneous pain in men. Sex differences in inflammation-induced pain sensitivity need further exploration. An intriguing mechanism for a potential sex difference was recently suggested in a murine study (217), where female mice did not require microglia activation to develop pain hypersensitivity, but appeared to have alternative routes *via* the adaptive immune system. This alternative route did not seem accessible to males. Future research will have to establish if these mechanisms are relevant for humans as well and their role in immune-driven pain sensitivity. Furthermore, sex-dependent alterations in neuroendocrine function in human subjects following LPS provocation have been shown (218). Healthy humans exhibited enhanced circulating levels of cortisol (peak response at 5 h post-LPS injection) after LPS injection (208, 219). The effect appears to be more pronounced in women (208), but the data are inconclusive (219). On a final note, experimental pain is sensitive to stress, which could potentially be a confounder in LPS studies on pain. Perhaps, surprisingly, however, stress levels generally remain low among the participants throughout the studies (207). Our experience is that because LPS stimulations, due to ethical considerations using bacterial endotoxin injections in healthy subjects, require very clear participant information and a hospital environment with experienced personnel and constant supervision, participants describe a feeling of safety and control even at higher, quite uncomfortable doses (such as 2.0 ng/kg).

Brain Activity during Experimental Immune Activation

Although the cytokines released during immune activation may affect and sensitize peripheral nerve endings, the main effect by which the immune system changes the function of the nervous system during sickness is believed to occur centrally *via* induced sickness behavior. It is reasonable to assume that changes in the emotional circuitries underlying the increased anxiety and depressed mood seen during immune activation may also lead to increased pain sensitivity due to overlapping function with the medial (affective) pain network (215), such as the amygdala, the cingulate, and prefrontal cortices. Also, as sickness is per definition an interoceptive signal, i.e., a signal of the internal state of the body (220), areas involved in interoception and homeostasis

such as the insular cortex, which is also part of the pain network, could potentially be affected. Several studies have attempted to elucidate the neural correlates of sickness behavior in the human brain. Most studies have used functional magnetic resonance imaging (fMRI) with cognitive and emotional paradigms. The main methodological limitation for this type of research is the fact that only the lower LPS doses used in humans are compatible with a brain scanning protocol, i.e., those that do not induce nausea or shivering.

Only two studies have explored pain perception directly during brain imaging so far, one using visceral pain stimuli (deep pain measurement) and mechanical pinprick pain (cutaneous pain measurement) (221) and the other using pressure pain (deep pain) (222). Benson et al. (221) showed increased activation within the posterior insula, dorsolateral PFC, anterior midcingulate, and somatosensory cortices for visceral pain stimulation, but not mechanical pain provocation. These areas are involved in pain and affective processing, interoception, and homeostatic regulation. Karshikoff et al. (222) described decreased activity after LPS injection in the lateral PFC and rostral anterior cingulate cortex (ACC), areas involved in descending pain inhibition, which may point to an increase in inflammation-induced pain sensitivity *via* diminished endogenous pain regulation. Additionally, the LPS group showed increased pain-dependent activity in the anterior insular cortex compared to placebo.

Emotional and cognitive fMRI paradigms corroborate the involvement of the cingulate, insula, and prefrontal cortices when the brain adapts to immune activation (221, 223–227), which are core areas in affective pain processing and pain regulation. Using a vaccination protocol as experimental immune provocation, Harrison et al. have shown increased activity in the subgenual ACC during emotional stimuli and in areas involved in interoceptive function during a Stoop task, such as the brain stem, the cingulate, and anterior insula (225, 226). To maintain the same level of performance during peripheral inflammatory activity, regions of the PFC appear to be required (224, 225)—areas implicated in pain regulation and processing of affective components of pain. In several studies, the increased BOLD activity in these areas correlates with peripheral cytokine levels (210, 222, 226, 228, 229).

Immune challenge affects the levels of neurotransmitters in the brain (6, 230). The expression of sickness behavior can potentially be manipulated by drugs affecting neurotransmitter levels such as serotonin reuptake inhibitors, which are compounds often used to ameliorate chronic pain. Hannestad et al. (231) have, for example, shown that the effects on fatigue are ameliorated by pretreatment of serotonin reuptake inhibitors, but not by dopamine and noradrenaline reuptake inhibitor. Peripherally induced inflammation has also been shown to activate microglia directly (232, 233). This is of special importance for chronic pain, as microglia have been implicated in the establishment of chronic pain (121).

In the past decade, it has thus been shown that acute inflammation induces pain sensitivity in humans as well. Most importantly, acute inflammation has a global effect on brain function, modulating the neural function in several brain areas involved in pain perception. Although the experimental models used are of

an acute character, similar mechanisms are likely to be involved when the organism is subdued to long-term inflammatory activity.

THE HPA SYSTEM AND PAIN IN ADULT ORGANISMS

Pain is not only modulated by immunological stressors but also by activation of the HPA axis. Pain is a sensory as well as an emotional experience. It is by nature a stressful event and, therefore, capable of activating the HPA axis. As we have mentioned, there is a large individual variability in developing chronic pain. One possible mechanism that may account for this individual variability in pain responses is how each individual responds to stressful events. Exaggeration or maladaptive response following stress may lead to altered pain responses. The HPA axis involves a defined neural circuit that comprises many brain regions including the amygdala, the mPFC, and the hippocampus. These areas are also important in pain modulation (234–237). In other words, a non-painful stressful stimulus is able to recruit parts of the same neural network involved in the pain response. Therefore, under conditions of stress, pain sensitivity may be exaggerated. Indeed, activation of CRH receptors in the amygdala facilitated pain responses through increased excitatory postsynaptic current in the parabrachio-amygdaloid synapse in rodents (238). Furthermore, administration of CRH into the CeA increased visceral nociception, as indicated by exaggerated number of abdominal muscle contractions in response to colorectal distension (239). On the other hand, the contribution of acute stress in analgesia commonly known as “stress-induced analgesia” has been traditionally well documented (240, 241), and at this point in time the exact contribution of cortisol in modulating pain is still a matter of debate within the scientific community.

In human clinical samples, some researchers have found that low back pain and enhanced musculoskeletal pain are often associated with hypocortisolemia (242, 243), while others demonstrated that patients suffering from chronic back pain displayed higher levels of cortisol compared to control group (244). This hypercortisolemia was associated with smaller hippocampal volume and higher pain-evoked response in the anterior parahippocampal gyrus (244). This variability in cortisolemia in pain condition not only may be due to the intensity of the stress response (245) but may also well depend on the neural circuit recruited following the stress stimulus, as the neural circuits within PVN are quite complex, and the final outcome depends on the nature of the stressor [for review, please see Ref. (237)]. In inflammatory pain model, such as the formalin test in rodents, LPS-induced hyperalgesia in infant and preadolescent rats coincided with increased circulating corticosterone 1 h following intraplantar injection of formalin (68). However, a recent study demonstrated that elevated levels of plasma corticosterone produced analgesia *via* attenuated C fiber-mediated spinal responses (246).

Overall, the abovementioned animal and human studies suggest that changes in HPA axis activity can contribute to pain. Although more studies are needed to confirm the exact contribution of cortisol (in humans) or corticosterone (in rodents) in modulating pain responses, the involvement of neuroendocrine

response in pain is evident. Therefore, new therapeutic approaches, which not only target neural activity but also the neuroendocrine axis, are needed to treat chronic pain patients.

A LIFETIME PERSPECTIVE

Although the acute effects of immune provocation on pain sensitivity are fairly well documented by now, as described in the previous sections, long-term inflammatory effects are not well understood. At this point in time, the most research on long-term effects of inflammatory activity on behavior has focused on depression. In humans, one incentive to study the mechanisms of sickness behavior came from clinical observations of immunotherapy eliciting side effects that resemble sickness behavior, such as depressive symptoms, fatigue, and aches. In, for example, hepatitis C patients undergoing IFN- α therapy, up to 45% of the patients develop depression (247). The typical signs of sickness behavior appear at the commencement of immunotherapy, whereas the establishment of depression requires time, and potentially, persistent inflammatory input during this time. It is now argued that depression is in part an inflammatory disease (248), and that a subgroup of clinically depressed patients suffers from a chronic low-grade systemic inflammation. Childhood trauma has also been shown to predispose persons to depression, but potentially not only *via* learning and HPA dysregulation as traditionally suggested but also *via* inflammation. Depressed patients with a history of traumatic events have higher low-grade inflammatory activity (249). Most interestingly, these patients benefit from pharmacological treatments that combine anti-inflammatory compounds and traditional antidepressants (249). Suggested mechanisms between inflammatory activity and depression include cytokines, serotonin, HPA dysregulation, GABA, and glutamate, all of which are neuroimmune pathways also implicated in pain [for extensive reviews see, e.g., Ref. (6, 250)]. Recent research is now shifting the focus toward similar mechanisms for chronic pain and fatigue (6, 230).

Inflammatory Disease and Pain

Chronic pain is a common comorbid symptom to many inflammatory diseases (251). Moreover, coronary heart disease (252), metabolic disorders (253), and life stress (254) increase the risk of developing chronic pain. It has been suggested that one of the underlying mechanisms for this association is indeed inflammation (252–254). Furthermore, chronic pain has been associated with low-grade inflammation (255). Mechanistically, peripheral chronic inflammation may become chronic within the CNS *via* changes in the central immune responses, by means of mechanism previously discussed. In animals, transient peripheral infections and inflammations or chronic exposure to low level (subclinical) inflammations can either activate microglia directly (256, 257) or “prime” the cells so that a recurrent inflammatory provocation becomes more severe (258). A systemic inflammatory challenge leads to an exaggerated fever response and sickness behavior in the presence of “primed” microglia in rodents (259, 260). “Priming” of immune components, or the requirement of a “second immunological hit” to reveal susceptibility as discussed

previously, is exemplified in a recent clinical study. Obesity has been associated with chronic pain and is considered a chronic low-grade inflammatory state (253). Obesity did not predict postsurgical pain intensity or inflammatory levels (255). BMI did, however, correlate with the increased immune response of leukocytes after LPS stimulation, suggesting sensitivity to inflammatory development in the obese patients that could result in complications associated with inflammation further down the road, such as chronic pain. Another study points to differences in pharmacological treatment strategies on pain after surgery, depending on prior inflammatory disease. Non-steroidal anti-inflammatory drugs had a better protective effect against the development of long-term pain after surgery in patients with a background of inflammatory disease, than opioids (261).

The Immune System Develops throughout Life

As discussed previously, the immune system carries the imprint of early-life inflammatory events. However, the function of immune system in fighting previously unencountered pathogens and protect the organism on reinfection relies on the ability to adapt and learn throughout life. Immune functioning is determined partly by genetics (262) and varies greatly between individuals. Individuals differ in their susceptibility to different types of infections, such as bacterial, viral, and fungal (262), and several single nucleotide polymorphisms related to immune pathways have been described (262). For example, the IL-6 and IL-8 pathways appear to have large genetic variations between individuals, while the IL-1 pathway has remained more conserved throughout evolution (262). Recent research emphasizes the importance of experience in shaping the adult immune response, similar to what has been described for infants in the previous sections. In fact, most of the individual differences seen in immune function in adult humans stem from non-heritable changes (263, 264). The immune system activates distinct cytokine patterns depending on the type of infection, and continuously learns from experience to adapt its inflammatory response (265). In theory, each person thus possesses an immune system that is a product of the types, strengths, and number of infections, diseases, and injuries encountered throughout life. Prior experience should thus impact future immunological reaction patterns. Epidemiological studies on comorbidity and risk factors for common disease give support to the idea that lifetime immune challenges affect disease susceptibility. A recent study shows that in patients with multimorbidity (in this specific study more than 10 disease diagnoses), the incidence of lifetime infections, inflammation, injuries, and tumors was 7–10 times as common as in a primary health care population (266). Lifetime accumulation of strong immune activation may thus potentially lead to increased general disease susceptibility and comorbidity (266). Furthermore, lifetime inflammatory disease is a risk factor for developing neurodegenerative disease (267–269). A plausible mechanism is that the accumulation of inflammatory activity in the body induces neuroinflammation in the brain, which in turn affects the function of the CNS (267).

When Adaptation Becomes a Liability

The process of perinatal programming posits that exposure to environmental factors during a sensitive window of development is able to program or have long-term consequences on physiological systems later in life. A fundamental aspect of perinatal programming is that developing organisms “sense” the early-life environment and use this information to establish homeostatic set points (270, 271). This process of perinatal programming has evolved as an adaptive mechanism enabling the fetus to constantly interact with the maternal environment (*via* the placenta) and use this information as a forecast of the environmental conditions it will eventually face postnatally. As such, preparing it to adjust its physiological and behavioral need to match the requirements of the *ex utero* world (272). In this perspective, fetal programming is an example of predictive adaptive responses where the fetus uses present cues to shape an adaptive phenotype to future environmental stimuli (31, 273). However, this adjustment can become maladaptive in the case where a “mismatch” exists between the expected *ex utero* environment and the actual circumstances. More importantly, when adverse events occur during a critical window of vulnerability of physiological systems that are still undergoing fine-tuning and plasticity, an individual may become predisposed to high susceptibility and exaggerated sensitivity to environmental stimuli later in life.

Correspondingly, the immune system and the HPA system adapt and change according to the stressors that the individual encounters throughout life, in order to maintain health and homeostasis. Pain is one of the most important survival signals available to us, and a life without pain perception is often a short one, as can be seen in individuals with congenital insensitivity to pain (274). However, when the imprint of the different stressors throughout life accumulate, interact, and/or become prolonged, the consequence may be detrimental for the pain system. For diseases like chronic pain, with such wide individual variability in symptomatology and treatment efficacy (8, 9), not only should comorbid disease and stressful life events (*i.e.*, concurrent with the pain) be considered when exploring the pathophysiology but also past stressors. In this study, we want to increase the awareness of the profound effect of the immune system on the pain system from birth to old age, *via* neuroimmune and neuroendocrine interactions. In other words, the faith of the pain system starts *in utero*.

CONCLUSION

In this review, we argue that the individual differences in the susceptibility to chronic pain and success of treatment thereof may be the result of the person's prenatal history, combined with childhood as well as lifetime experience. We have highlighted the biological underpinnings and potential consequences on the pain system induced by the stress and infectious/inflammatory load an individual is subjected to. The neuroimmune and neuroendocrine interactions that affect the pain system start in the womb and modulate the pain system throughout life. The modulations may be of both structural and functional nature and may be both adaptive and maladaptive. In order to understand individual

differences in pain, human studies of long-term effects of inflammatory stressors are needed.

AUTHOR CONTRIBUTIONS

IZ and BK wrote the manuscript and approved the final version.

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Targeting the Brain Reservoirs: Toward an HIV Cure

Céline Marban¹, Faezeh Forouzanfar², Amina Ait-Ammar², Faiza Fahmi², Hala El Mekdad^{2,3}, Fadoua Daouad², Olivier Rohr^{2,3,4*} and Christian Schwartz^{2,3*}

¹INSERM UMR 1121 Faculté de Chirurgie Dentaire, Université de Strasbourg, Strasbourg, France, ²EA7292, DHPI, Université de Strasbourg, Strasbourg, France, ³IUT Louis Pasteur de Schiltigheim, Université de Strasbourg, Schiltigheim, France, ⁴Institut Universitaire de France, Paris, France

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Ilhssane Zouikr,
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Japan

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Taisuke Izumi,
Frederick National Laboratory for
Cancer Research, USA
Carine M. C. Van Lint,
Université libre de Bruxelles, Belgium

*Correspondence:

Olivier Rohr
olivier.rohr@unistra.fr;
Christian Schwartz
schwartz.christian@unistra.fr

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One of the top research priorities of the international AIDS society by the action “Towards an HIV Cure” is the purge or the decrease of the pool of all latently infected cells. This strategy is based on reactivation of latently reservoirs (the shock) followed by an intensifying combination antiretroviral therapy (cART) to kill them (the kill). The central nervous system (CNS) has potential latently infected cells, i.e., perivascular macrophages, microglial cells, and astrocytes that will need to be eliminated. However, the CNS has several characteristics that may preclude the achievement of a cure. In this review, we discuss several limitations to the eradication of brain reservoirs and how we could circumvent these limitations by making it efforts in four directions: (i) designing efficient latency-reversal agents for CNS-cell types, (ii) improving cART by targeting HIV transcription, (iii) improving delivery of HIV drugs in the CNS and in the CNS-cell types, and (iv) developing therapeutic immunization. As a prerequisite to these efforts, we also believe that a better comprehension of molecular mechanisms involved in establishment and persistence of HIV latency in brain reservoirs are essential to design new molecules for strategies aiming to achieve a cure for instance the “shock and kill” strategy.

Keywords: brain, reservoirs, latency, cure, cART, HIV transcription

INTRODUCTION

Combination antiretroviral therapy (cART), introduced in 1996, has radically improved the management of HIV-1 infection and decreased both morbidity and mortality. However, despite initial hopes to cure HIV, treatments were unable to fully eliminate the virus (1–3). Indeed, with very sensitive methods (4–6), a remaining viremia is always noticed in patients on cART. Moreover, HIV RNA returns to a measurable plasma level when cART is disrupted (7, 8). The origin of this persistent viremia is still a matter of debate (9–11). Latent persistence of HIV in long-lived cells, such as the central memory CD4+ T-cells, hematopoietic stem cells, dendritic cells, and cells from the monocyte-macrophages lineage in the form of proviruses have been described (1, 2, 12–19). Moreover, these cells are located in a variety of anatomical sites, including tissues, such as blood, brain, gut-associated lymphoid tissue, bone marrow, and genital tract (20), making it difficult to purge the virus from all the reservoirs.

These latently infected cells are from time to time reactivated and produce HIV particles at low levels, thus explaining the persistence of viremia. An alternative theory, the cryptic ongoing replication states that despite cART, HIV is continuously produced at low levels. The inefficiency of the treatment in cells supporting ongoing replication could be due to poor drug penetration in sanctuaries, such as the brain (21) or by cell-to-cell transfer of the virus (22). In theory, there are critical

therapeutic implications for cART as it is expected that during ongoing replication, drug resistance might arise (23–26). The potential mechanisms of HIV persistence have been discussed recently in a review by Hong and Mellors (27).

One of the main debates in the field of HIV reservoir is whether or not the central nervous system (CNS) constitutes a real viral reservoir. Indeed, with its unique features, such as the existence of a blood–brain barrier (BBB) with poor drug penetration, the CNS might be considered as a sanctuary (20) made of specific cell types (28) with reduced immune surveillance. Moreover, the anatomy of the CNS is such that there is poor viral genetic information exchange with the other sites and, thus, might be referred as a compartment (20, 29, 30).

First, we will give our opinion on the existence of viral reservoirs in the CNS referring to excellent recent reviews in this topic. Next, we will discuss the importance to purge these potential viral reservoirs. Indeed, in theory, it is possible to acquire virus resistance to cART if there is an ongoing replication in the brain. Another major concern is the existence of HIV-associated neurocognitive disorders (HAND). In up to 50% of the HIV-infected patients on efficient cART and undetectable virus load (≤ 50 copies/ml), HAND has been recorded. Several mechanisms are evoked to explain the increase of less severe forms of HAND in which production of some viral proteins occurs during reactivation or cryptic ongoing HIV replication. We will then review the state of art of what is known regarding the molecular mechanisms underlying the establishment and persistence of HIV in the potential reservoirs in the brain and, finally, discuss the profound therapeutic implications of purging reservoirs.

CAN THE CNS BE QUALIFIED AS A HIV RESERVOIR?

A viral reservoir is an infected cell population that allows persistence of replication-competent virus in patients under cART (20). According to this definition, the only true reservoirs are the resting CD4⁺ T-cells. Indeed, these cells fulfill all the criteria to be considered as a real reservoir, i.e., presence of integrated virus in long-lived cells, persistence of high levels of virus in a quiescent/latent state in the reservoir and possible reactivation of the virus with the formation of replication-competent particles (31).

There are several evidences that brain cells harbor genome-integrated HIV (28). We know that the virus invades the brain very soon following infection. Virus infection was shown in astrocytes (32), in perivascular macrophages (32), and in microglial cells (33). All three cell types are long-lived cells with perivascular macrophages (34) and astrocytes (35) with a half-life ranging from months and microglial cells with a half-life of years (36). All these cells are infected at high frequency in the brain. Astrocytes, the most abundant cell type in the brain, are infected in up to 19% of the cell population (37). Similar ratio of infected cells has been found among the perivascular macrophages and the microglial cells (33, 38). In addition, several mechanisms, including epigenetic regulation, have been evoked to induce latency in these cells notably in astrocytes and microglial cells (39–42).

Due to ethical and technical problems, it is not possible to evaluate the human brain-infected cells for their capacity to produce replication-competent viruses. However, there are several indirect evidences showing that CNS is a reservoir for HIV. Indeed, HIV DNA has been detected in brain tissues isolated from autopsies of HIV patients whose infection has been controlled by cART (33, 39). Moreover, there is a strong correlation of the amount of HIV DNA found in astrocytes and HIV-associated dementia (HAD) (37). Various animal models have been used to show persistence of HIV infection in the CNS as brain biopsy is not possible. Indeed, several animal models, such as macaque, rats, and humanized BLT mouse, have been used to mimic the condition of HIV-infected patients on cART, which confirmed the presence of viral RNA or viral proteins in the brain (43–45). Specifically, in the macaque model, a mechanism of the establishment of transcriptional HIV latency in the CNS has been suggested (46). They notably showed that interferon beta repressed SIV LTR activity by inducing C/EBP γ expression, a dominant negative isoform of C/EBP β (47). There are also several evidences supporting continuous CNS perturbation despite an efficient cART (48) with an increase of the prevalence of milder form of HAND. Moreover, in patients under suppressive cART activation of the immune system is still observed in the CNS with some biomarkers, such as neopterin or NFL being detected in the cerebrospinal fluid (CSF) (49). One explanation is the existence of an inflammatory process that might be driven by low-level HIV replication in infected cells (50, 51). Interestingly, neuroimaging data are also in favor of persistent CNS inflammation in patients on cART (52, 53). Finally, development of highly sensitive methods, such as single-copy assay (SCA), has allowed the detection of HIV RNA in the CSF from infected patients on cART or from elite controllers whose HIV RNA level was initially undetectable in the plasma and CSF (54–56). The recent discovery of a CSF viral escape in patients on cART with undetectable plasma HIV RNA but with neurological impairment argue also for the existence of a persistent HIV reservoir in the brain (55–59). In conclusion, there are now several evidences supporting that CNS is a reservoir for HIV even if it is still controversial. Readers will be referred to the following reviews that nourish the debate of whether or not CNS serves as a HIV reservoir (60–63).

WHY IS IT IMPORTANT TO PURGE THE CNS RESERVOIR OF HIV?

The CNS is involved in the control of most functions of the body and mind. The brain operates in a very well controlled microenvironment separated from the other parts of the body by two barriers: the choroid plexus and the BBB. The two barriers, but predominantly the BBB, constitute physical barriers and any perturbation of their integrity will be associated with neurological diseases. There are several other features that make the CNS unique. The CNS has specific immunological features; principally an innate immune response through the perivascular macrophages and the microglial cells. However, the adaptive immune response has also been observed and, thus, contributes to the immune surveillance in the CNS (64, 65).

Indeed, leukocytes trafficking to the CSF either by traversing the BBB to the perivascular space or the choroid plexus has been detected (66). More interestingly, in patients having CSF/plasma HIV discordance (patients having higher levels of HIV RNA in CSF than in blood) even at very low levels it was demonstrated that both innate (macrophages and microglial cells) and adaptive (T CD4+ and CD8+ lymphocytes) are involved in CNS injury (67–70). It has been shown that the percentage of a specific set of T CD8+ lymphocytes that expresses interferon γ is higher in the CSF than in blood. Moreover, this higher percentage of T CD8+ cells in CSF versus blood contributes to the occurrence of HAND (67) [reviewed in Ref. (71)]. Within 2 weeks following acute infection by HIV, the virus enters the CNS. There are at least two mechanisms to explain how HIV crosses the BBB, including trafficking of cell free virus and infected cells (72). The well-documented infection of the CNS is accomplished through infected cells and, thus, has been named the “Trojan Horse” mechanism (73). A recent study using natalizumab, an anti- $\alpha 4$ blocking antibody preventing both lymphocytes and monocytes trafficking across the BBB, is in accordance with this mechanism. Indeed, a drastic decrease of SIV DNA in the brain was observed when natalizumab was given to rhesus macaque during acute SIV infection (74). According to this theory, infected monocytes cross the BBB and infect the perivascular macrophages, the microglial cells, and the astrocytes that result in HIV-associated neurological disorders (75). Since the introduction of cART, an important decrease in the incidence of the severe form of HAND has been noticed (76). However, there is an increase of milder form of the infection (up to 50%), which might be largely under diagnosed. Thus, better screening tools to detect HAND are required in the future (77). The reasons for the increase of the prevalence of milder forms of HAND are not fully understood. One explanation might be related to the existence of quiescent/latent viral reservoirs in the CNS that emphasizes the importance of eradicating the reservoirs. Another major concern related to the existence of such quiescent/latent reservoirs in the CNS is that it might be a source of new particles that could replenish the periphery blood. These notions will be discussed in the later chapters.

HIV-1 and HIV-Associated Neurological Disorders

HIV-associated neurocognitive disorders have been divided into three subgroups according to the Frascati criteria, i.e., asymptomatic neurocognitive impairment (ANI), mild neurocognitive disorder (MND), and HAD (78). These disorders are associated with the entry of HIV into the CNS that occurs almost immediately after systemic infection (79). The more severe form of HAND, i.e., HAD has drastically decreased with the introduction of cART. However, the less severe forms (MND and ANI) have continued with a prevalence ranging from 20% to up to 50%, while keeping in mind that these milder forms are often under diagnosed (80, 81). However, the details of persistence of these less severe forms of HAND in patients on cART are not fully understood. There are at least two hallmarks of HIV infection in the brain, i.e., chronic immune activation and compromised BBB integrity in which the central role for HIV neuropathogenesis

is played by the monocytes/macrophages (82–85). Importantly, immune activation still occurs in patients on cART (50, 51). The exact mechanisms of such pathogenesis are not entirely known and rely on two models: a direct and an indirect model (86, 87). In the direct model, infected cells will cause neuronal death through the action of newly synthesized viral proteins, such as Tat, gp120, Vpr, and Nef. The two major viral proteins that lead to neuronal injury are Tat and gp120. Their effects are mediated through their interaction with neuronal cell receptors, such as the NMDA receptor and the chemokine receptors (CCR5 and CXCR4). More details on the mechanisms involved in the neuropathogenesis caused by viral proteins are found in the review (88). In the indirect model, sustained chronic inflammation is induced by secreting perivascular macrophages, microglial cells, and to a lesser extent by astrocytes releasing neurotoxic host factors. Among these secreted products, there are proinflammatory cytokines (TNF α , IL-1 β , IL-6, IL-8, and INF α), chemokines (CCL2 and CCL5), and small molecules, such as quinolinic acid and the platelet-activating factor. Moreover, these viral proteins and cellular factors increase the oxidative stress and alter the integrity of the BBB which in turn results in the stimulation of even more infected cells in the brain. Further investigations are needed to decipher the exact mechanisms involved in CNS injury. Interestingly, Tat might be involved in both direct and indirect processes that lead ultimately to neuronal death. Potential roles and functions of Tat in both direct and indirect neurotoxicities have been described elsewhere (89, 90). The importance of Tat is still a matter of debate since there are controversies regarding the amount of Tat present in the CNS cells environment and the amount of Tat used in *in vitro* experiments. In favor of its importance is the use of Tat transgenic animal model where CNS injury has been observed (91, 92). Therefore, it will be essential to detect Tat in the brain from patients on cART. It is possible that this protein might arise from quiescent/latent reservoirs and, therefore, be responsible for the milder form of HAND. Improvement of cART by targeting the production phase of HIV-1, including transcription appears, therefore, crucial (93). Indeed, current cART is not targeting this step and since the CNS infection occurs almost immediately during acute infection, establishment of infected reservoirs will not be prevented. Moreover, strategies aiming to purge the reservoirs are based on HIV reactivation with the risk that viral proteins, such as Tat will be produced in the brain. HIV-1-mediated neuropathogenesis might also involve a dynamic interaction between astrocytes and peripheral blood mononuclear cells (PBMCs) (94). Indeed, a recent report showed that astrocytes susceptibility to produce HIV infection is enhanced by PBMCs producing interferon γ which in turn inhibit HIV-1 production in PBMCs through the secretion of small glycoprotein, i.e., the Wtns. These later proteins have been shown to be involved in many CNS processes (95), such as synaptic plasticity and neurotransmitter release, which might explain partly HIV-1-mediated neuropathogenesis.

CNS Reservoirs as a Source of Virus

The CNS has two special features making it difficult the achievement of a cure. First of all, the CNS is considered as

a sanctuary for HIV by pharmacologic means as it is a site with limited access to antiretroviral drugs (ARV) (96–99). As an outcome, there is a risk to allow the occurrence of virus resistant to the current drugs used in cART. Second, the CNS is also considered as a compartment in which the virus is isolated from other parts of the body (29, 100). Because of poor genetic information exchange with the other sites, neurotropic variants of HIV might be selected, which most likely will not respond to treatment in a similar way than the virus encountered in the CD4⁺ T-cells, the main target in the body. There are now numerous evidences supporting the fact that the CNS-resident virus has evolved to become macrophage tropic (101). Indeed, sequence analysis of the *env* gene and of the HIV-1 promoter (LTR) argue for the compartmentalization of HIV variants in the CNS (102–105). Variations in the promoter are important since mechanisms involved in the establishment and persistence of latency in the CNS might differ from the one described in CD4⁺ T-cells. As mentioned above, this will impact the efficiency of latency-reversing agents (LRA) in strategies aiming to purge the latent/quiescent reservoirs (106, 107). Another major concern regarding the necessity to purge the CNS reservoirs is related to the discovery of CNS viral escape in patients on cART (108). Initial studies have shown occasional cases of virus escapes in the CSF (109, 110). Development of highly sensitive assays has even allowed the detection of CSF HIV RNA, which were not detectable with previous assays (111). Indeed in a report, evaluation of CSF viral escape has been done in a cohort of neurologically asymptomatic patients successfully treated with cART. It was shown that around 10% of these patients had detectable CSF HIV RNA, suggesting that viral escape may be underestimated (112). The recent discovery of a CNS viral escape in a cohort of 14 patients on cART with undetectable plasma HIV RNA but who developed HIV-encephalitis argues for the possibility that CNS is a real reservoir (57). Actually, this study and others raise the question that CNS-specific viral replication can occur in patients on cART from reactivated reservoirs which in theory may have escaped therapy and ultimately lead to drugs resistance (58, 59, 113). Very interestingly a similar drug-privileged site, i.e., the lymphoid tissue has been shown to have low access to drugs (114). The authors notably showed that the virus is continuously produced and might be a source of HIV from which replenishment of blood occurs. However, and contrary to the brain, they do not show that resistance to antiretroviral drugs arises. The authors of this study suggest that this absence of resistance to ARV might be explained by the too low level of drug concentration in lymphoid tissue that is not sufficient to confer competitive advantages to the development of drug-resistant viruses. This study point out to the importance of developing new ways to deliver drugs in all sanctuaries, including brain and lymphoid tissues (115).

Overall, we suggest that it is crucial to eradicate brain reservoirs since ARV-resistant viruses are capable to replenish the systemic circulation from these reservoirs. It will also imply that CSF analysis in patients on cART should be performed more often since it will greatly help assessing the compartmentalization of HIV in the brain and monitoring the efficiency of new

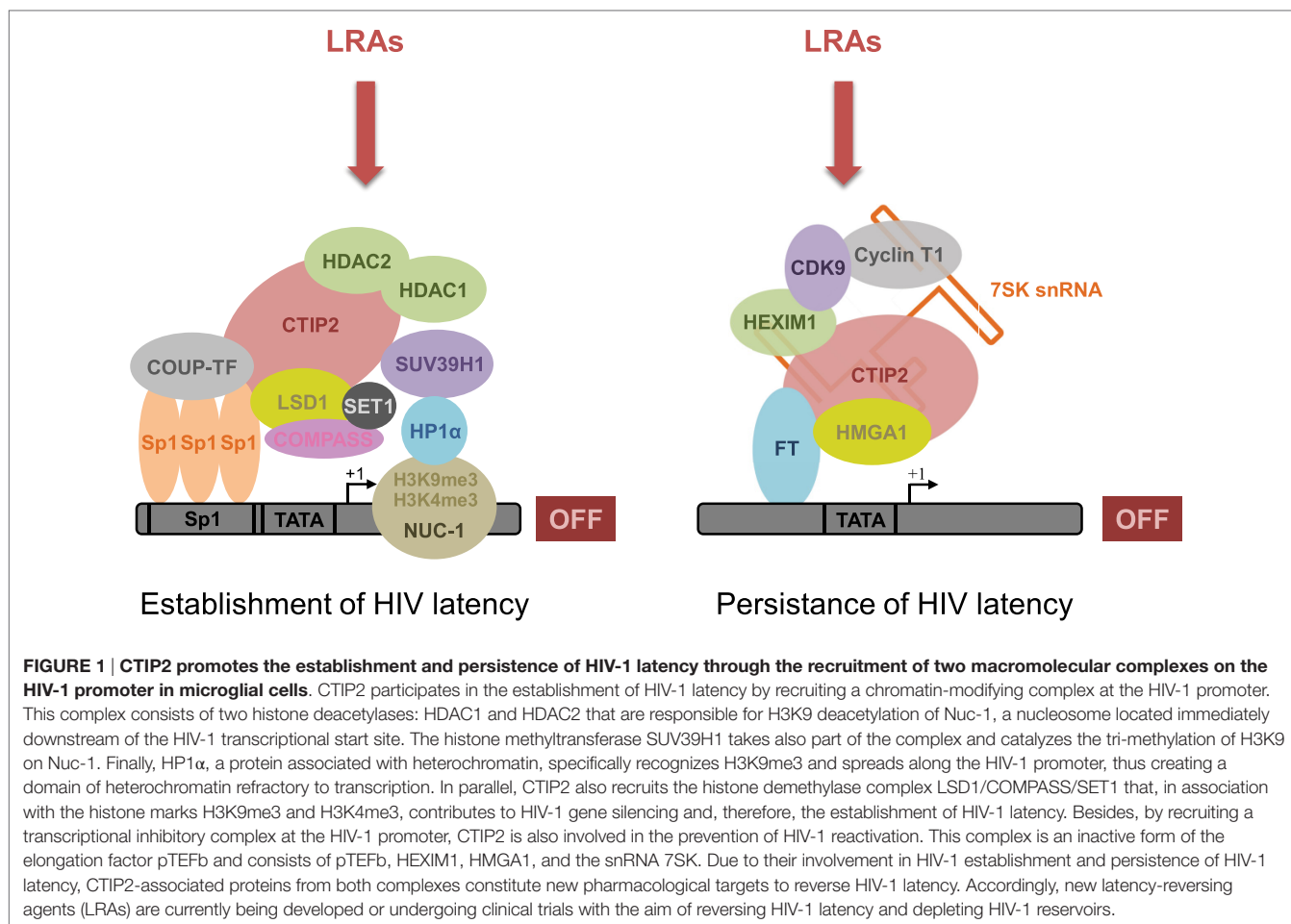
treatments (116). Notably, CSF might be used to evaluate HIV drug resistance.

MOLECULAR MECHANISMS OF HIV-1 LATENCY

Establishment and persistence of HIV latency occur in brain cells, i.e., perivascular macrophages, microglial cells, and astrocytes. Infection of these cells differs from the infection of blood cells infected, mainly the CD4⁺ T-cells. Indeed, HIV infection in macrophages is not lytic and these cells are far more resistant to cytopathic effects. Moreover, infected monocyte–macrophage cells are also more resistant to apoptosis, a major obstacle for the eradication of the virus. These cells may harbor latent viruses for months (perivascular macrophages) or for years (microglial cells). Astrocytes are also thought to be infected by HIV-1 despite the lack of the co-receptors CCR5 and CXCR4 probably through the involvement of vesicles (38). However, the infection appears to be non-productive with only early transcripts, such as *tat* and *nef*, that are detectable at very low level (117).

Understanding the intimate mechanisms underlying HIV-1 latency in these CNS-specific cells is necessary to develop new and original therapies for viral eradication. The molecular mechanisms underlying these therapies are determined by the cellular specificity of HIV gene transcription and the variability of the LTR found in viruses having evolved in the brain (61, 118). For example, it has been shown in microglial cells that Sp3 and a truncated form of C/EBP β (NF-IL6) inhibit the basal transcriptional activity of HIV-1 (47). Such a reduced basal and Tat-activated transcriptional activity has also been shown in astrocytes. Transcriptional silencing has been associated with low levels of TAR RNA binding proteins (TRBP) and with mutations of the SP motifs found within the LTR of brain-derived HIV-1. Mutations prevent the transcription factor Sp1 to bind the promoter and, thus, inhibit transcriptional activation (119, 120). However, the main mechanism involved in establishment and persistence of latency involves epigenetic regulation (41, 121, 122). In our laboratory, we showed that the cellular factor COUP-TF interacting protein (CTIP2) is a key factor in the establishment and persistence of HIV latency in microglial cells (123). We notably showed that this protein serves as a platform to anchor several protein complexes having different functions. Indeed, at least two different complexes containing CTIP2 are involved in the establishment and the persistence of HIV-1 latency (**Figure 1**). Moreover, CTIP2 is also involved in the control of cellular genes of importance for the virus. Among these factors, the cellular cyclin-dependent kinase inhibitor CDKN1A/p21^{waf} has been described to favor HIV-1 gene transcription in the monocyte–macrophage lineage. This effect indirectly favors HIV-1 latency since activation of the p21 gene stimulates viral expression in macrophages (124). Moreover, CTIP2 counteracts HIV-1 Vpr protein that is required for p21 expression (125). We, therefore, suggested that CTIP2 generates a cellular environment disfavoring viral reactivation and, thus, favoring HIV-1 latency.

The first CTIP2-associated complex described in our laboratory has been involved in the establishment of HIV-1 latency through the induction of heterochromatin in the vicinity of the



viral promoter (**Figure 1**, left complex). Indeed, we showed that CTIP2 recruits a chromatin-modifying complex through the Sp1 sites of the proximal promoter (42). This complex contains the histone deacetylases HDAC1, HDAC2, and histone methyltransferase SUV39H1 that specifically demethylates lysine 9 of histone H3. This histone modification allows heterochromatin protein 1 (HP1) binding, heterochromatin formation, and hence HIV silencing (42, 126, 127). In a consecutive study, we have shown that CTIP2 interacts physically and functionally with the lysine-specific demethylase (LSD1) to repress HIV-1 transcription and viral expression in a synergistic manner (128). The recruitment of LSD1 at the HIV-1 proximal promoter has been associated with both H3K4me3 and H3K9me3 epigenetic marks, which is linked to the recruitment of hSet1 and WDR5, two members of the hCOMPASS complex, on the HIV-1 promoter (128). Recruitment of CTIP2 on the p21 promoter also induces a heterochromatin environment. Moreover, CTIP2 has been shown to silence p21 gene transcription by creating epigenetic marks of repression, as described above for the HIV-1 promoter (125). Interestingly epigenetic regulation of HIV-1 latency, which was associated with the recruitment of HDACs and SUV39H1 has also been described in astrocytes (40). Finally, in a recent report, investigation of the neuropathology and the molecular alterations

associated with CNS latent HIV-1 infections provided evidence that HIV-1 persistence in the brain is associated with high level of CTIP2, HDACs, and HP1 (39).

We also showed that CTIP2 belongs to another complex able to prevent HIV-1 reactivation (**Figure 1**, right complex) (129). Indeed previous work has shown that CTIP2 represses the late phase, Tat-dependent, of HIV-1 transcription (127). In the absence of the trans-activator factor Tat, an inactive form of the elongation factor pTEFb is found in a multiprotein complex, including 7SK snRNA, CTIP2, and HEXIM1 anchored to viral and cellular gene promoters (129). pTEFb is composed of a regulatory subunit CyclinT1 and a catalytic subunit CDK9, whose kinase activity is involved in the Ser2 phosphorylation of the carboxyl terminal end of the RNA polymerase II and in the phosphorylation of the negative transcriptional elongation factors NELF and DSIF. Following phosphorylation, the RNA pol II processivity significantly increases, which leads to an efficient transcription of genes (130). Interestingly, we have shown that CTIP2 drastically repressed CDK9 kinase activity in this inactive complex, thus, inhibited pTEFb function. Finally, we showed that the cellular protein high mobility group AT-hook 1 (HMGA1), which also belongs to the 7SK snRNA complex recruits the inactive CTIP2/pTEFb complex to the HIV-1 and cellular target

promoters (131). As a consequence, protein complexes containing CTIP2 regulate viral and endogenous gene expression, thus favoring HIV-1 persistence. Far more investigations are still needed to decipher the precise molecular mechanisms involved in these processes. We still do not fully understand how the transition from transcription initiation into elongation (which involves pTEFb) is controlled by cellular factors and/or the viral transactivator Tat. We and others hypothesized that the inactive form of the pTEFb complex is part of a 7SK complex that is anchored to the promoter by either CTIP2 (129) or Kap1 (132), thus available for RNAPII elongation through its activation. The transition from the inactive to the active form of the pTEFb complex through the action of Tat is not well understood but may involve a phosphatase (PPM1G/PP2C γ) that takes apart pTEFb from the 7SK complex (133).

THERAPEUTIC IMPLICATIONS FOR THE ERADICATION OF HIV-1 FROM BRAIN RESERVOIRS

Several considerations already mentioned [emergence of multidrug resistance (24, 113), non-AIDS-related events (134–136) etc.] urge the search of new ways to develop a sterilizing or a functional cure for AIDS (137). The purge of viral reservoirs by the “shock and kill” strategy (138) is a possible approach to achieve such a cure. This strategy aims at purging or at least reducing the size of cellular reservoirs by reactivating HIV transcription (shock) followed by intensive cART therapy and immune activation (kill) (139, 140). As several reports suggested, using LRA alone or in combination have proven the efficiency of this strategy in the reactivation of quiescent/latent HIV from CD4⁺ T-cells reservoirs (138, 141–145). Several clinical trials have been carried out and some others are in progress or forthcoming (146). This strategy of reactivation needs to work on all potential reservoirs, including brain reservoirs. However, several limitations to the eradication of the brain reservoirs may preclude a cure.

Limitations to the Eradication of Brain Reservoirs

It is essential to decipher the molecular mechanisms underlying HIV persistence in all types of potential reservoirs, since some important differences in those mechanisms have been noticed in all latently infected cell types. For example, LSD1 has been associated with activation of HIV transcription in CD4⁺ T-cells (147). However, in microglial cells, LSD1 played a role in the establishment of latency (128). LSD1 mediates HIV-1 transcription silencing in microglial by anchoring various factors at the promoter rather than inducing HIV-1 transcription by its own enzymatic activity in CD4⁺ T-cells. The dual role of LSD1 achieved by different mechanisms in the two main HIV-1 cellular targets points to the complexity of the molecular mechanisms of HIV latency (148). Hence, additional investigations of the epigenetic regulation of HIV latency are needed in order to develop efficient drugs targeting each potential viral reservoir. Furthermore, as mentioned in the previous sections, there are

several characteristics of the CNS, which limits a cure by the “shock and kill” strategy:

- i. The CNS is a sanctuary with barriers (BBB and choroid plexus) that reduce the access of some of the drugs currently used to the brain (97).
- ii. The main cellular targets are astrocytes and CNS-resident macrophages. However, few drugs are able to target the monocyte–macrophages lineage (149) and the effects of cART on HIV replication in astrocytes are unknown or neurotoxic (150).
- iii. CNS has long been considered as an immunologically privileged site (151). Therefore, achieving immune activation through cytotoxic T lymphocytes (CTL) activation to eliminate the potential reservoirs may be difficult or even deleterious in the brain.
- iv. Another major concern is related to the fact that reactivation of the virus with LRA will lead to the synthesis of neurotoxic viral proteins, such as Tat and the gp120, as there are no drugs currently available targeting HIV transcription. Moreover, reactivation of the virus is often associated with CNS inflammation through macrophage/microglial cell activation (152, 153).

How Can We Overcome These Limitations?

With these limitations evoked in the previous section, it may be difficult to achieve a purge in the CNS. The idea is to eliminate or reduce the pool of latent/quiescent reservoirs with the aim to mimic elite controllers able to control the HIV infection and with very low amount of reservoirs. Introducing cART very early following HIV infection has been proved to be efficient since it limits the size of the latent/quiescent reservoirs (154–156).

In our opinion, achieving a sterilizing cure or a partial functional cure in the brain needs efforts in four directions: (i) designing efficient LRA for CNS-cell types, (ii) improving cART by targeting HIV transcription, (iii) improving delivery of HIV drugs in the CNS and in the CNS-cell types, and (iv) developing therapeutic immunization.

Designing potent LRAs to reactivate HIV-1 transcription from the CNS-cell types is crucial in a “shock and kill” strategy. However, we and others have shown that the molecular mechanisms involved in the establishment and persistence of latency in these cells may differ from the mechanisms involved in the CD4⁺ T-cells that are currently the main targets for LRAs (106, 107, 137). As a consequence, the outcome in the use of LRAs may differ in CNS-cell types. Several HDAC inhibitors (HDACi) have been tested in the U1 monocyte cell line and in primary cells (astrocytes and macrophages) (106, 107, 157). Preliminary data showed that some LRAs, including panobinostat (158) and JQ1 (159), are relatively non-toxic and efficient to induce HIV reactivation at a therapeutic concentration (106, 107). On the contrary, other LRAs, including disulfiram and vorinostat, which were promising in CD4⁺ T-cells, were not working at therapeutic concentration in the CNS-cell types (106, 107). Among LRAs, bryostatin-1 is very promising since it can cross the BBB to activate brain Protein Kinase C especially in the two main targets

for HIV-1, i.e., microglial cells and astrocytes (142, 160). This PKC activator has already been used in both preclinical trials for Alzheimer disease and in clinical trials to treat cancers [reviewed in Ref. (161)]. Further investigations will be needed to characterize new targets, such as the hCompass complex, recruited on the viral promoter by LSD1 in microglial cells. Preclinical studies in animal models are also needed to test the efficacy of LRAs. Combinations of LRAs have to be tested *in vitro* and *in vivo* as well, since they may work in a synergistic manner as described (142, 162). Using combination of LRAs with lower dose may also prevent some drug side effects when used alone at a higher concentration [reviewed in Ref. (163)]. Finally, a recent pilot study has suggested that administration of panobinostat, a potent activator of HIV transcription in CNS-cell types, was not associated with side effect in the brain as assessed by CSF biomarkers, such as neopterin, C reactive protein, and IP-10 (164).

Improving cART by targeting HIV transcription is also crucial since there are currently no drugs targeting this step (93). Moreover, reactivation of HIV leads to the synthesis of neurotoxic viral proteins, such as Tat. We and others discussed in details the importance of targeting this step and readers are referred to these recent reviews (93, 165). Particularly, inhibitors may be developed against the two main targets that control HIV transcription, i.e., the cellular factor NF-KB and the viral transactivator Tat. Since NF-KB also plays a central role in inflammation, new drugs targeting this factor will also prevent or at least reduce chronic inflammation in the brain (166, 167). It is also important to target the viral transactivator Tat since this factor is involved in the regulation of HIV-1 and its secreted form induces neuronal death by direct neurotoxicity. Several molecules, especially natural compounds deserve attention (168, 169). We believe that characterization of new targets associated with the exploitation of new technologies, such as bioengineering, high-throughput screening, computer-aided drug design, and combinatorial chemistry, will considerably improve the discovery of new drugs. Among the molecules that deserve attention, we can mention the dCA, a chemical derivative of corticostatin, a natural steroidal alkaloid isolated from a sponge (170–172). A promising Tat inhibitor has been recently isolated from the plant *Tripterygium wilfordii* and named triptolide (173). This molecule, which is currently in phase III of a clinical trial, inhibits both HIV replication and transcription by increasing the proteasomal degradation of Tat. Another family of protein that deserves attention is the DING proteins (pDINGs), a family of potential therapeutic agents against HIV-1 (174–178). These molecules discovered in bacteria, plants, and animals have been reported to inhibit HIV transcription. In addition, it has been shown that a phosphorylated form of pDING is a neuroprotective factor and could be used to reduce neuro inflammation due to HIV-1 (88, 179).

Another major limitation to purge brain reservoirs is related to the poor access of the drugs in the CNS due to the presence of barriers, such as the BBB. Moreover, drugs have to target macrophages and astrocytes. Indeed, it has been shown that all drugs, except protease inhibitors, display reduced activity in macrophages compared to CD4+ T-cells (180). We have already mentioned that some LRAs have no effect in the CNS-cell types at a therapeutic concentration. Different mechanisms have

been evoked to explain the lower EC50 values of these drugs in macrophages/microglial cells. Drug penetration may be reduced due to the differential expression of efflux transporter and multidrug resistance proteins (181, 182). Several ways are explored to overcome these limitations and discussed in other reviews (183, 184). Improvement of both bioavailability and bio-distribution of drugs used in cART will increase the access of these drugs to the brain. Among the approaches used to improve drug delivery in the brain, there is the development of carriers, such as liposomes, dendrimers, and micelles. A particularly promising approach is based on polymeric nanomedicines that raise hope for eradication of HIV from all potential reservoirs [reviewed in Ref. (185–187)]. Increase in treatment efficacy and tolerance may be expected, hence favoring patient adherence. These later strategies may also increase the distribution of drugs in CNS-cell types, such as astrocytes and macrophages/microglial cells. Indeed, macrophages/microglial cells constitute an important but neglected barrier for HIV eradication, which will need efforts to circumvent (149, 188). Several conventional and new therapeutics against HIV-1 in macrophages, including PI3K/Akt blocking agents, carbohydrate-binding agents, and small interfering RNAs, have been discussed elsewhere and deserve real attention (184, 189).

Immune-based therapeutics should also be considered since the size of the reservoir following treatment with LRAs is not reduced and need immune activation to clear them (190). In particular, CTL activation has been shown to clear HIV-1 from infected CD4+ T-cells (140). Previous studies done in animal models argued in favor of the importance of CTL in the clearance of HIV-1 infected macrophages in the brain (191, 192). A CD8+ T-cell response appears essential in the control of other brain infections, such as toxoplasmosis (193). Dealing with immune activation is not easy and constitutes a challenge for strategies aiming to eradicate HIV-1 reservoirs. These approaches need further investigations and development of adequate animal models to ensure the feasibility of such treatments (194, 195). Another unexplored non-conventional way to clear reactivated latently infected cells is based on the use of neutralizing antibodies against HIV-1 with promising results obtained in humanized mice (143, 196).

CONCLUSION

Reducing the size of reservoirs is fundamental for HIV+ patients to control their viral replication without any treatment, a situation typical for elite controllers. The purge of HIV reservoirs constitutes, therefore, one of the top research priority of the International AIDS Society (IAS) through the action “Toward an HIV Cure.” We may expect to get a sterilizing cure by eradicating the virus from all the reservoirs but a more realistic view would be a functional cure through the reduction of the pool of cellular reservoirs. A major problem is to reduce/eradicate reservoirs located in the CNS. There are now numerous direct and indirect arguments for the existence of a pool of quiescent/latent reservoirs in the brain even if it has not been demonstrated in human yet. The strategy called “shock and kill” enables reactivation of quiescent/latent reservoirs

followed by an intensive cART to clear the reservoirs. Several pilot clinical trials have been done and some are ongoing and upcoming. The results of trials are encouraging but also point to the need of additional interventions, such as immune activation, in order to clear the reservoirs. This immune activation approach is needed to eliminate or reduce brain reservoirs but might be difficult since the CNS has several unique characteristics. Indeed, the CNS is a pharmacological sanctuary and a compartment isolated from the other parts of the body. In addition, latently infected cells in the brain, i.e., astrocytes and macrophages/microglial cells are rather different from the main memory T-cells reservoir. Altogether, intense efforts are needed in several directions, including the design of efficient LRAs for CNS-cell types, improving cART by targeting HIV transcription, improving delivery of HIV drugs in the CNS and in the CNS-cell types and developing therapeutic immunization therapies in order to overcome the above discussed limitations. We believe that we are at a crossroads to achieve a cure for HIV. Indeed, there are several adequate animal models (non-human primate, humanized mice, etc.) to test the efficiency of strategies aiming to purge reservoirs. Identification of new targets and the availability of new technologies will also allow the design of new original drugs. In particular, new natural compounds and their derivatives could help in the design of

new class of molecules targeting HIV-1 transcription a step not yet targeted by cART. This is especially crucial in a strategy aiming to reactivate latent CNS-cell types. Finally, hope rises also with the advent of nanotechnologies. Although still in the early stage, nanotechnologies could be used in drug transport to enable drugs to reach both the brain (by crossing barriers such as the BBB) and the CNS-cell types (by crossing cell membranes). Dosage is expected lower and in consequence less toxicity and a better adherence to treatment is awaited.

AUTHOR CONTRIBUTIONS

CM revised the manuscript and made substantial contributions to its final content and design. AA-A, Ffo, FFa, FD, and HM revised the manuscript. OR and CS drafted the manuscript. All authors read and approved the final manuscript.

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Emerging Roles for the Immune System in Traumatic Brain Injury

Celia A. McKee and John R. Lukens*

Department of Neuroscience, Center for Brain Immunology and Glia, School of Medicine, University of Virginia, Charlottesville, VA, USA

Traumatic brain injury (TBI) affects an ever-growing population of all ages with long-term consequences on health and cognition. Many of the issues that TBI patients face are thought to be mediated by the immune system. Primary brain damage that occurs at the time of injury can be exacerbated and prolonged for months or even years by chronic inflammatory processes, which can ultimately lead to secondary cell death, neurodegeneration, and long-lasting neurological impairment. Researchers have turned to rodent models of TBI in order to understand how inflammatory cells and immunological signaling regulate the post-injury response and recovery mechanisms. In addition, the development of numerous methods to manipulate genes involved in inflammation has recently expanded the possibilities of investigating the immune response in TBI models. As results from these studies accumulate, scientists have started to link cells and signaling pathways to pro- and anti-inflammatory processes that may contribute beneficial or detrimental effects to the injured brain. Moreover, emerging data suggest that targeting aspects of the immune response may offer promising strategies to treat TBI. This review will cover insights gained from studies that approach TBI research from an immunological perspective and will summarize our current understanding of the involvement of specific immune cell types and cytokines in TBI pathogenesis.

Keywords: traumatic brain injury, neuroinflammation, cytokine, inflammasome, innate immunology, neuroprotection, microglia, neurodegeneration

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*Correspondence:

John R. Lukens
jrl7n@virginia.edu

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INTRODUCTION

Traumatic brain injury (TBI) affects millions of people worldwide every year, and current estimates from the World Health Organization (WHO) suggest that TBI will be the third leading cause of death and disability by the year 2020 (1). In the US alone, upwards of 1.7 million Americans seek medical treatment for some form of brain trauma each year (2, 3), and nearly 2% of the American population, or approximately 5–6 million people, currently suffer from TBI-related disabilities (4). TBI is a particularly serious threat to health in newborns, children, the elderly, military service personnel, and athletes involved in contact sports. Trauma to the brain can result in persistent and debilitating impairments in cognition, sensory function, mental health, and motor function. Furthermore, TBI-induced inflammation and pathology have been strongly linked to increased risks of developing numerous neurological disorders including anxiety, depression, PTSD, Alzheimer's disease (AD), chronic traumatic encephalopathy (CTE), Parkinson's disease, and amyotrophic lateral sclerosis (ALS) (5–10).

Despite being a prevalent and pressing global medical issue, there are currently no FDA-approved therapeutics to treat TBI. In recent years, mounting evidence from both TBI patients and animal

models of brain injury implicate dysregulated immune responses in the potentiation of TBI-induced neurological dysfunction and brain pathology (11–16). For instance, elevated cytokine production is one of the strongest prognostic indicators of poor clinical outcomes in TBI (17–21), and brain trauma has been shown to induce immune-mediated inflammatory responses that can last for years post-injury (22, 23).

In addition to providing vital protective measures against pathogens and tumors, the immune system is also centrally involved in the restoration of tissue homeostasis following injury. Critical functions that are carried out by the immune system in response to injury include the sequestration of tissue-damaging irritants, engulfment and disposal of cellular debris, and the promotion of the wound-healing response. Tissue damage that results from trauma, ischemia-reperfusion injury, metabolic distress, and environmental irritants provokes the release of damage-associated molecular patterns (DAMPs) [e.g., ATP, reactive oxygen species (ROS), damaged mitochondria, and necrotic cells] and alarmins [e.g., interleukin (IL)-1 α , IL-33, HMGB1]. The recognition of DAMPs and alarmins by immune receptors then stimulates the local production of cytokines and chemokines at the site of injury, which subsequently coordinates the activation, expansion, and recruitment of immune cells to areas of tissue damage.

Brain trauma results in two phases of tissue injury. The first round of injury is a direct result of exorbitant mechanical impact to the brain tissue. The aftermath of a severe blow to the head results in immediate neuronal and glial cell death, axonal injury, disruption of the blood–brain barrier (BBB), edema, and the release of DAMPs and excitotoxic agents (24). The immune response to TBI is intended to promote neuroprotection and repair, but can become maladaptive if dysregulation occurs. Whether the immune response contributes to repair or further destruction ultimately depends on the nature, duration, and magnitude of the immunological events that develop in response to brain injury. If not properly controlled, the immune system can provoke a secondary phase of tissue damage and neuroinflammation. In contrast to the acute nature of the primary brain injury, the secondary tissue damage generally results in a diffuse, long-lasting injury. The fundamental role that the immune system plays in driving the secondary phase of tissue damage following brain trauma has led many to believe that immunomodulatory approaches may offer a much-needed strategy to treat TBI. In this review, we discuss how aspects of the immune response can influence clinical outcomes following TBI. In particular, we highlight recent findings from experimental models of TBI that define central roles for individual immune cell types and cytokines in TBI pathogenesis (**Figure 1**; **Table 1**).

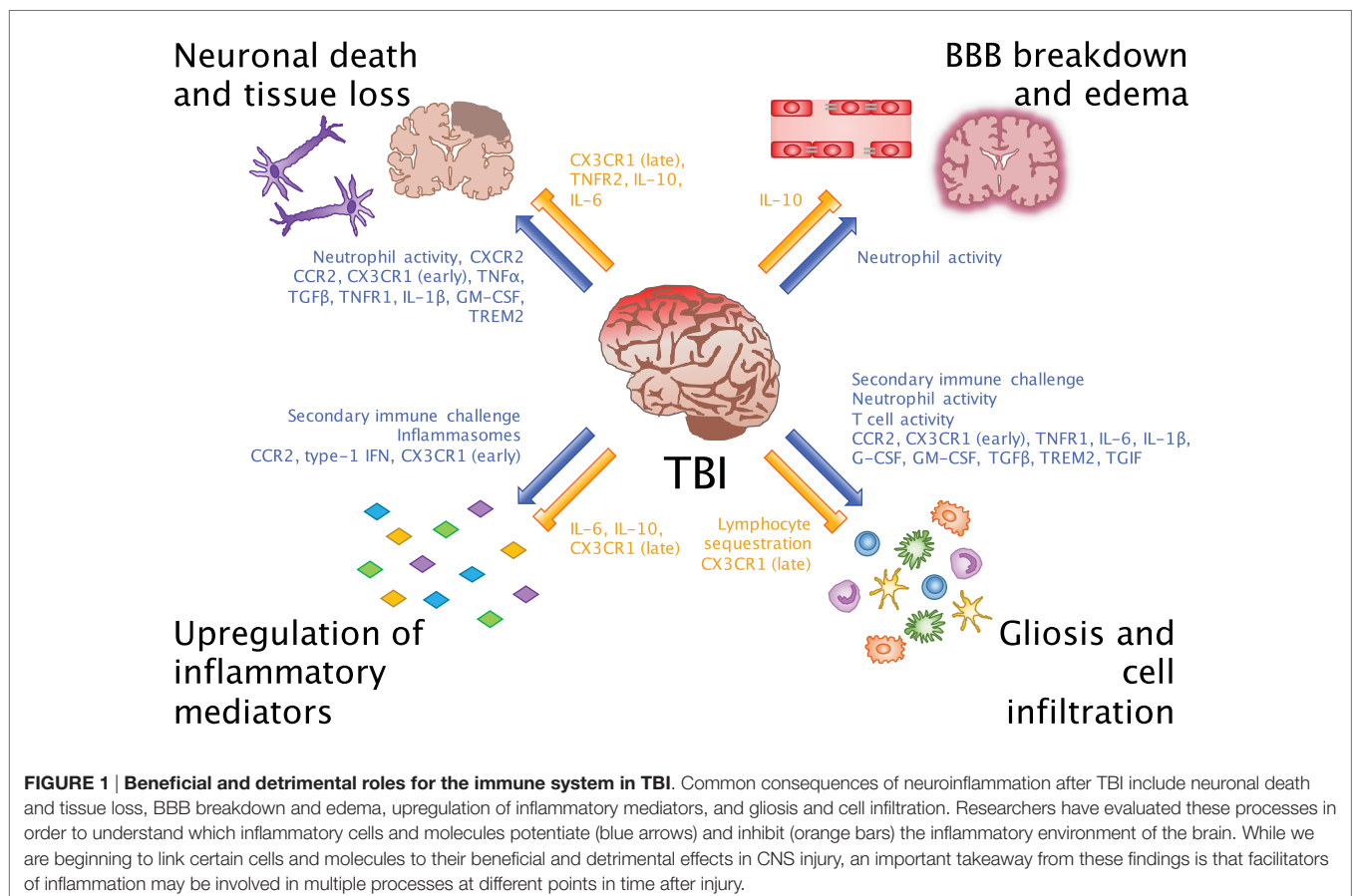


FIGURE 1 | Beneficial and detrimental roles for the immune system in TBI. Common consequences of neuroinflammation after TBI include neuronal death and tissue loss, BBB breakdown and edema, upregulation of inflammatory mediators, and gliosis and cell infiltration. Researchers have evaluated these processes in order to understand which inflammatory cells and molecules potentiate (blue arrows) and inhibit (orange bars) the inflammatory environment of the brain. While we are beginning to link certain cells and molecules to their beneficial and detrimental effects in CNS injury, an important takeaway from these findings is that facilitators of inflammation may be involved in multiple processes at different points in time after injury.

TABLE 1 | Key immune mediators involved in TBI pathogenesis.

Cell types	Mediators	Functions
Neutrophils	CXCR2 (C–X–C motif chemokine receptor 2) NE (neutrophil elastase)	Chemokine that mediates neutrophil migration Enzyme released by neutrophils to degrade extracellular matrix
Macrophages and microglia	CD11b (cluster of differentiation 11b) CCR2 (C–C motif chemokine receptor 2) CX3CR1 (C–X3–C motif chemokine receptor 1) IBA1 (ionized calcium-binding adapter molecule 1)	Integrin that regulates migration of immune cells through tissues Chemokine receptor that coordinates monocyte chemotaxis Chemokine receptor mediating macrophage and microglia migration Calcium-binding protein associated with microglia and macrophage activation
T cells	Rag1 (recombination activating gene 1) IL-4 (interleukin 4)	Enzyme that is required for B and T cell development Cytokine that aids in B and T cell proliferation and differentiation
Others	IL-1 (interleukin 1) Caspase-1 IL-18 (interleukin 18) IL-6 (interleukin 6) GFAP (glial fibrillary acidic protein) TNF α (tumor necrosis factor α) G-CSF (granulocyte colony-stimulating factor) GM-CSF (granulocyte-macrophage colony-stimulating factor) Type 1 IFN (type 1 interferon) IL-10 (interleukin 10) TGF- β (transforming growth factor β) TREM2 (triggering receptor expressed on myeloid cells 2)	Pro-inflammatory cytokine that regulates transcription and production of multiple downstream inflammatory mediators Enzyme that cleaves pro-IL-1 β and pro-IL-18 to induce inflammation Pro-inflammatory cytokine that activates NK and T cells Pleiotropic cytokine that induces a multitude of inflammatory responses Intermediate filament protein expressed by astrocytes Pleiotropic cytokine that can promote cell death, inflammatory cytokine production, and cell proliferation Stimulates proliferation and differentiation of hematopoietic cells as well as neural progenitors Promotes generation and activation of myeloid cells and neurons Regulates transcription of pro-inflammatory cytokines and chemokines Negatively regulates pro-inflammatory cytokine production Controls proliferation and differentiation of multiple immune cell types Activates myeloid cells upon sensing lipoproteins, may be involved in debris removal and cell survival

THE KINETICS OF THE IMMUNE RESPONSE TO BRAIN INJURY

Upon brain injury, DAMPs and alarmins are released into the extracellular space where they can then signal through pattern-recognition receptors (PRRs) and cytokine receptors on CNS resident cells. This promotes the production of cytokines and chemokines that are involved in coordinating the recruitment of immune cells to sites of tissue damage (**Figure 2**). Neutrophils are the first immune cells that are recruited to the brain in response to trauma (25, 26). They first appear in the sub-arachnoid and vascular space surrounding the site of tissue damage within hours of injury. Neutrophils then begin to infiltrate into the brain parenchyma at 24 h post-injury (27). As the first responders, they play critical roles in the containment of the injury lesion and in the removal of cellular debris and damaged cells. Neutrophils predominate during the first days following injury; however, their numbers diminish greatly between days 3 and 5 post-injury. This time point coincides with the recruitment of other peripheral immune cells and the local activation of microglia and astrocytes. CCR2-expressing monocytes are the major immune cell population that infiltrates into the damaged tissue at days 3–5 post-injury, although T cells, natural killer (NK) cells, and dendritic cells (DCs) can also be detected around the injury site (15, 28–30). The coordinated production of chemokines following trauma orchestrates the recruitment of immune cells to areas of brain injury. The major chemokine pathways that are involved in mobilizing immune cells to brain damage have been comprehensively reviewed recently (11, 31–33) and will not be covered in great detail in this review. By 2 weeks post-injury, the

brain is largely devoid of any infiltrating immune cells. However, activated microglia and astrocytes and elevated levels of inflammatory cytokines can be detected for months to years following brain injury (22, 23, 34, 35). This is unlike what is seen following tissue damage in other peripheral organs, where tissue resident macrophages and stromal cells typically return to a resting or immunologically quiescent state within weeks post-injury. The existence of activated glial cells and aberrant regulation of cytokine expression for months to years post-TBI suggests that the immune response to TBI can persist for long periods beyond the initial trauma.

INVOLVEMENT OF IMMUNE CELL TYPES IN TBI

Neutrophils

Considering their role in vascular permeability and edema in peripheral tissues, neutrophils have largely been implicated in BBB breakdown and edema in TBI. However, it is still unclear how their activity is related to these processes. Early TBI papers agree that neutrophils can accumulate at sites of injury within hours post-injury (26, 36) and that the number of neutrophils that are recruited to sites of brain trauma typically correlates with the severity of the injury (37). However, studies on the role of neutrophils in mediating BBB breakdown, edema, and neurodegeneration have been inconclusive. Although neutrophilia has been reported to coincide with BBB breakdown and neurodegeneration, these processes seem to be disconnected spatially and temporally from the invasion of neutrophils (26). Furthermore,

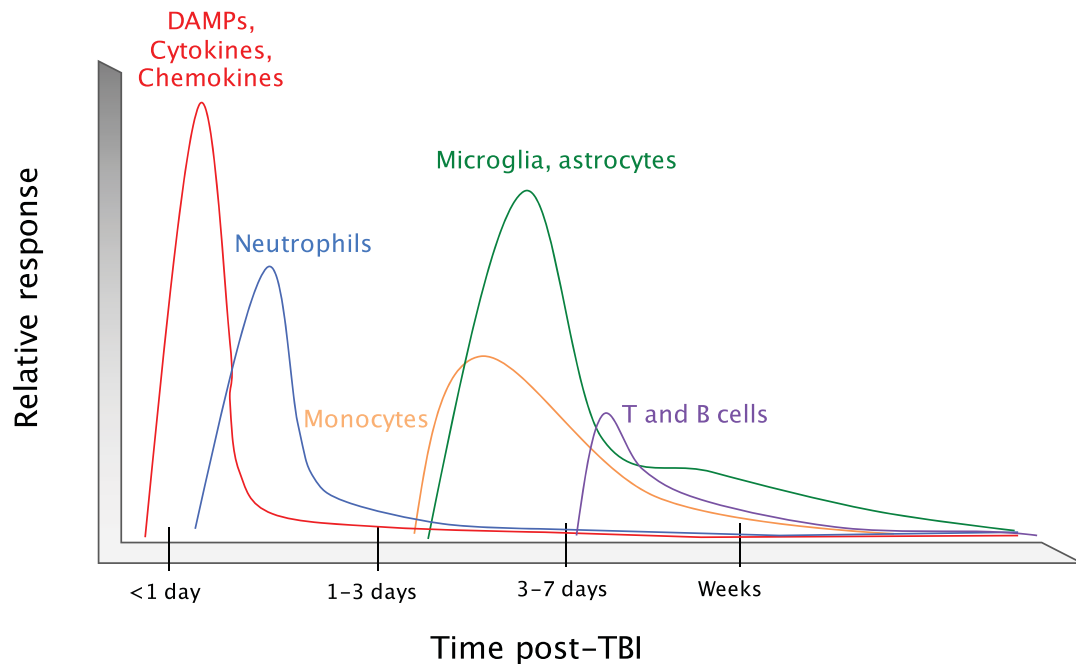


FIGURE 2 | Timeline of the immune response to TBI. Upon an impact to the head, cellular damage results in the rapid release of damage-associated molecular patterns (DAMPs) that prompt resident cells to release cytokines and chemokines. These signals quickly call in neutrophils, which aid in the containment of the injury site and promote the removal of debris and damaged cells. As neutrophil numbers begin to decline after a period of days, infiltrating monocytes and activated glia begin to accumulate around the site of injury to perform reparative functions. Depending on the severity of the brain injury, T and B cells can also be recruited to sites of brain pathology at later time points in the response (3–7 days post-injury).

efforts to deplete neutrophils have been unsuccessful in linking them to loss in BBB integrity (36), which was thought to be the event responsible for subsequent edema and neuronal death.

Due to these early findings, researchers began to think of neutrophil activity and tissue edema as having important consequences independent of BBB breakdown. Kenne et al. used an anti-Gr-1 antibody to deplete neutrophils in a cortical controlled impact (CCI) model and found that neutrophil depletion led to decreased edema for at least 48 h after injury, but did so without ameliorating BBB permeability (38). Neutrophil depletion was associated with decreased numbers of apoptotic cells, reduced macrophage/microglia activation in the cortex, and mitigated tissue loss. These data are similar to results from CXCR2 knockout mice, which were used by Semple et al. to reduce CXCR2-mediated infiltration of neutrophils after TBI (39). These mice did show reduced neutrophil infiltration into the brain, but BBB breakdown appeared similar to wild-type mice. While they also showed significantly less cell death within the lesion, this did not have an impact on functional outcome. Collectively, these two studies suggest that neutrophil depletion may have neuroprotective effects in TBI without necessarily being linked to BBB breakdown.

Further research into the importance of neutrophil activity post-TBI has begun to characterize the mechanisms involved in neutrophil-mediated neurodegeneration at early time points. For instance, Semple et al. used neutrophil elastase (NE) knockout mice in a CCI model to investigate how neutrophil effector

functions contribute to secondary tissue damage and neurological dysfunction following brain trauma (40). They found that NE-deficient mice exhibit significantly diminished edema at 24 h after injury. However, this was not associated with reductions in neutrophil numbers or decreased production of matrix metalloproteinase-9 (MMP-9), which is known to regulate neutrophil migration by promoting extracellular matrix breakdown and/or through the modulation of chemokine activity. NE knockout mice also had reduced numbers of apoptotic neurons as well as lower heme-oxygenase levels in the hippocampus at 24 h after injury, indicating attenuated cell death and a less severe oxidative state. However, these early neuroprotective effects did not prevent cortical or hippocampal volume loss in the long-term, which may explain why NE deficiency was not found to improve behavioral performance at 2 months post-injury. These findings suggest that NE activity contributes to injury-induced edema and early neurodegeneration.

Thus, it is becoming clearer that neutrophils are linked to cerebral edema and neuronal death in TBI, but the relationship between neutrophil activity and BBB breakdown is not as clear as previously thought. It is likely that the distinct differences between the BBB and other vascular barriers outside the brain mean that the BBB structure has a different relationship with neutrophils that remains to be elucidated. In future studies, it will be important to investigate whether vascular edema in TBI is directly responsible for releasing cytotoxic substances that cause neuronal death following TBI or whether neutrophils and other

inflammatory cells within the parenchyma are the primary source of neurotoxic factors that promote cytotoxic edema and early neurodegeneration in TBI.

Macrophages and Microglia

There has been tremendous interest in defining the discrete roles of macrophages and microglia in TBI. Activated microglia and macrophages release pro- and anti-inflammatory factors that can signal to resident and peripheral cells to promote or resolve the inflammatory response to trauma. Chronically activated microglia and macrophages have been found in rodent models and humans after TBI (22, 23, 41, 42) and are considered to be one of the hallmarks of unresolved inflammation that may have long-term consequences (43, 44).

Groups have utilized different methods to deplete microglia and macrophages *in vivo* in order to characterize their roles in TBI-induced neuroinflammation, tissue damage, and neurological dysfunction. Two of these methods use targeted depletion of CD11b-expressing cells with transgenic CD11b-TK (thymidine kinase) and CD11b-DTR (diphtheria toxin receptor) mice (45, 46). While both methods were effective in reducing their target cell types post-TBI, neither attenuated signs of tissue damage such as axonal injury and lesion size. However, it should be noted that both of the treatment approaches that were used to deplete CD11b-expressing cells in these studies were found to cause inflammation even in uninjured mice. Therefore, it is likely that triggering inflammation before injury had an effect on the outcomes that were observed in these studies.

The chemokine receptor CCR2 plays critical roles in the recruitment of monocytes/macrophages to the brain, and, as a result, suppression of CCR2 signaling is often exploited to reduce the effects of infiltrating monocytes/macrophages in TBI studies. Numerous recent reports have shown that abrogating CCR2-mediated events can markedly limit both TBI-induced neuroinflammation and cognitive decline. For instance, Morganti et al. found that the CCR2 antagonist CCX872 reduces accumulation of peripheral macrophages in the brain and alters the regulation of several pro- and anti-inflammatory cytokines as well as NADPH oxidase (NOX2) production after CCI (47). These effects were associated with less severe hippocampal-dependent cognitive dysfunction. Similarly, CCR2 deficiency in another CCI study reduced numbers of infiltrating monocytes and rescued long-term spatial learning and memory deficits in the Morris water maze (MWM) test (48). Another group disrupted CCR2 activity by knocking in a red fluorescent protein (RFP) protein at the *Ccr2* gene locus in mice. In their studies, they found that impaired CCR2 signaling prevents monocyte recruitment into the brain and reduces cavity volume and axonal pathology following fluid percussion injury (FPI) (49). Taken together, these studies indicate that inhibition of CCR2-mediated cell infiltration limits neurodegeneration and neurological decline following brain trauma.

A recent study by Zanier et al. used CX3CR1 knockout mice to disrupt CX3CL1 chemokine signaling in order to understand its importance in controlling myeloid cell activity in TBI (50). After receiving a CCI injury, CX3CR1 knockout animals showed neurological protection 4 days following TBI. However, while

wild-type mice returned to pre-injury levels of neuroscore performance by 5 weeks post-injury, CX3CR1-deficient mice still exhibited appreciable impairments in neuroscore performance at this time point. This decline in neuroscore performance at later time points in *Cx3cr1*^{-/-} mice was associated with persistent neuronal death and an overall decrease in neuronal numbers. Further investigation into the effects of disrupted CX3CR1 signaling on macrophages and microglia showed that these cell types exhibit a more protective, anti-inflammatory phenotype in injured CX3CR1-null mice than seen in injured controls at early time points. However, at 5 weeks post-TBI, CX3CR1-deficient mice showed signs of elevated myeloid cell activation as compared to wild-type animals. Taken together, these results indicate that while early CX3CR1 signaling may have detrimental effects, this signaling is necessary at later time points post-brain injury to prevent long-term inflammation and cognitive impairment.

Another issue facing the TBI field is how to best define inflammatory cell types. Using principal component analysis (PCA) and microarray analysis of brain macrophages, Hsieh et al. found that the subset of macrophages expressing the M2 (alternatively activated macrophages)-associated marker arginase-1 (*Arg1*) had a distinctly different transcriptional profile from arginase-1-negative cells, but that the genes they expressed after TBI did not match traditional M2 markers (51). They found that while *Arg1*⁺ and *Arg1*⁻ macrophages expressed a variety of M1 and M2 markers, they differed distinctly in their chemokine profiles and several genes involved in injury protection and wound healing. These data indicate that delineating macrophages by an M1 (classically activated macrophages) or M2 phenotype in TBI obscures other macrophage subsets that may have distinct roles in the injury response.

Emerging data also suggest that macrophage phenotypes may be more flexible than once thought. Wang et al. set out to characterize the timeline of M1 and M2 macrophage/microglia activity after CCI. By tracking M1 macrophages/microglia with the marker CD16/32 and M2 macrophages/microglia with CD206, they found that at 3 and 7 days after injury the majority of *Iba1*⁺ cells assumed an M1 phenotype, yet at day 5 there was a rise in M2 macrophage/microglial cell numbers (52). This shift from an M1 state to an M2 phenotype and back may provide protection from possible detrimental effects of a prolonged state of either phenotype. The authors also found that white matter injury correlated with M1 cells, peaking at 3 and 7 days.

Clarifying the activation timeline and phenotypes of macrophages and microglia will likely be important in understanding how unresolved inflammation can lead to long-term detrimental consequences. An emerging body of literature is beginning to define how microglia and macrophages can be primed by TBI and generate exaggerated immune response and functional deficits upon secondary immune challenge. For example, injection of LPS at 30 days after injury in an FPI model induced more robust inflammatory cytokine production by CD11b-expressing cells in TBI animals than in controls (53). This was associated with decreased social exploratory behavior at 24 h after LPS injection as well as depressive behaviors. This same group found that secondary immune challenge also caused learning and memory

deficits that could be linked to TBI-mediated microglia priming (54). These data indicate that at long-term time points, when behavioral deficits appear to have normalized following brain injury, a second immune challenge can produce further cognitive decline.

Taken together, these studies provide examples of how the TBI field is beginning to characterize macrophage and microglia migration, activation, and priming in relation to functional deficits after TBI. An important consideration for this field is that many authors choose to study both macrophages and microglia as a combined population, acknowledging that it is difficult to distinguish them within an inflammatory context using current markers, such as CD11b, CD45, CX3CR1, and IBA1. However, considering the importance of these cells in both the short and long-term inflammatory states, more specifically targeted techniques would help to define their discrete roles. In addition, considering the timeline of their activation, adapting methods to study macrophage and microglia signaling over the acute and chronic phases of TBI will be necessary in order to uncover time-dependent beneficial and detrimental effects as well as identifying effective therapeutic windows.

T Cells

The kinetics of T cell infiltration have been described in TBI patients and animal models (55–57), but it still remains unclear what role(s) they play in brain trauma-associated wound-healing responses. In a study by Weckbach et al., *Rag1*^{-/-} mice were used to investigate how the absence of B and T cells influences brain pathology and neurological impairment following weight drop-induced TBI (58). Surprisingly, lacking the adaptive arm of the immune system did not appreciably affect neurological outcome, BBB integrity, pro- or anti-apoptotic mediators, hippocampal architecture, or astroglial activation in these studies.

In a separate study, Mencl et al. used the sphingosine-1-phosphate receptor agonist and lymphocyte sequesterer FTY720 to inhibit T cell migration to the brain following TBI (59). While FTY720 did decrease the numbers of circulating lymphocytes, it did not provide any protection to TBI animals in terms of lesion volume, neuroscore, apoptotic neurons, BBB maintenance, or edema. However, FTY720 was found to reduce the numbers of neutrophils and macrophages/microglia in the ipsilateral hemisphere at 1 day after injury. Thus, future investigations should evaluate the ability of T cells to regulate the infiltration of other immune cells into sites of brain injury.

In future studies, it will be important to move away from methods that promote global defects in T cell responses and consider more specific effects of T cell subsets on TBI progression. In other models of CNS injury, T cells have been found to confer neuroprotection (60–62). For example, Walsh et al. recently reported that protection after spinal cord injury (SCI) is guided by specific T cell-derived cytokines, particularly IL-4 (63). Their interest in IL-4 stemmed from the observation that T cells within the site of injury were the major producers of IL-4 in their model and that functional recovery was markedly delayed following SCI in IL-4 knockout mice. They found that reconstituting *Rag1*^{-/-} mice with IL-4-deficient T cells prior to injury did not lead to functional recovery, but transfer of

wild-type T cells did. In addition, a MyD88-dependent Th2 skew of T cells was necessary to produce IL-4 and induce elevated neurotrophin signaling and axonal outgrowth both *in vitro* and *in vivo*. This insight into T cell subsets in injury recovery may apply to the TBI field, and thus warrants more specific investigations.

INFLAMMATORY MEDIATORS IN TBI

Interleukin-1

Interleukin-1 is a potent pro-inflammatory cytokine that has been implicated in numerous inflammatory and neurological disorders. Secretion of IL-1 must be tightly regulated in the brain, as unchecked IL-1 production has been shown to provoke neuroinflammation and neurodegeneration. There are two distinct forms of IL-1 – IL-1 α and IL-1 β – both of which can induce similar levels of inflammatory signaling following engagement of IL-1 receptor (IL-1R). Although IL-1 α and IL-1 β evoke almost identical downstream inflammatory responses, their expression patterns and requirements for activation differ greatly. IL-1 α is constitutively expressed by all nucleated cells, and secreted full-length IL-1 α can transmit inflammatory signaling without the need for further modification or processing. In contrast, IL-1 β is generated as a biologically inactive pro-form protein that requires cleavage to elicit its inflammatory properties and secretion (64). Caspase-1 activation in inflammasome complexes has emerged as a major mechanism for both IL-1 β cleavage and IL-1 α release (14, 65–69), although recent studies have also begun to identify additional inflammasome-independent pathways that promote IL-1 production (64, 70–72).

Interleukin-1 β is one of the most frequently measured cytokines in the TBI literature, and it has been shown to be increased after TBI in humans and mouse models (46, 47, 53, 73–83). During neuroinflammation, IL-1 β is known to have profound effects on BBB permeability, glial activation, immune cell recruitment, and neurodegeneration (84–86) and is likely one of the first immune mediators as it peaks early after injury. IL-1 β is known to strongly enhance inflammatory responses following TBI, and this has led many to postulate that IL-1 production may negatively impact clinical outcomes following brain trauma (75).

Recently, progress has been made using methods to neutralize or antagonize the activity of IL-1 β in TBI. In two different studies, Clausen et al. administered an anti-IL-1 β neutralizing antibody to CCI-injured animals through 14 days after TBI (87, 88). In these studies, IL-1 β neutralization led to a decrease in the numbers of microglia/macrophages, neutrophils, and T cells in the brain, especially at 7 days after injury. Although they did not report appreciable differences in motor coordination performance during the rotarod test, they did observe better performance during learning trials in the MWM, as well as decreased tissue loss at experimental endpoints for anti-IL-1 β -treated animals.

In other models of CNS injury, IL-1 α upregulation precedes that of IL-1 β and IL-1 α deletion limits neuronal damage and promotes accelerated functional recovery (89). IL-1 α has also been suggested to jump start an inflammatory loop that is sustained and enhanced upon the upregulation of IL-1 β , accounting for the

excess of IL-1 signaling commonly seen in inflammatory states (90). Thus, future studies should investigate the effects of IL-1 α separately from IL-1 β , as their baseline expression, regulation, and secretion differ significantly and may thus control the kinetics of inflammation in different ways.

In humans, the recombinant IL-1 receptor antagonist anakinra is currently being tested to treat severe TBI, as it has shown some promise in the treatment of stroke (91). Helmy et al. used anakinra with PCA analysis to demonstrate that IL-1 signaling is a pivotal upstream regulator of TBI-induced cytokine production (92), which, they showed in a later trial, may lead to a shift in macrophages to express higher levels of pro-inflammatory cytokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-1 β (93). This result is somewhat surprising considering the antagonistic effect on IL-1 signaling anakinra would be expected to have, but it suggests that further exploration into the mechanisms involved as well as delineation of the distinct functions of IL-1 α and IL-1 β during neuroinflammation will likely yield critical insights into the regulation of TBI pathogenesis by IL-1 signaling.

As described briefly above, inflammasome signaling has emerged as a major mechanism involved in IL-1 production. Inflammasomes are multiprotein complexes that coordinate caspase-1-mediated inflammatory cytokine production and cell death. Recent studies have shown that aberrant regulation of inflammasome signaling is a major driver of inflammation and pathology in multiple models of tissue damage, including stroke, macular degeneration, and renal ischemia (16, 66, 73, 94, 95). Inflammasomes consist of a sensor molecule such as a Nod-like receptor (NLR) or a pyrin/HIN domain-containing protein (PYHIN) family receptor, the adaptor protein ASC (apoptosis associated speck-like protein containing a CARD), and caspase-1. To date, five receptors – NLRP1, NLRP3, NLRC4, AIM2, and PYRIN – have been discovered to promote inflammasome signaling. Following the detection of their cognate danger- or pathogen-associated triggers, inflammasome-associated NLRs and PYHIN family receptors promote rapid inflammasome complex formation. The coordinated assembly of this multiprotein inflammasome platform promotes activation-inducing auto-cleavage of caspase-1. Activated caspase-1 can then cleave both pro-IL-1 β and pro-IL-18, which is required to elicit their inflammatory properties and for their secretion. Bioactive caspase-1 also provokes pyroptosis, which is a gasdermin D-mediated inflammatory form of cell death that is associated with the release of the pro-inflammatory alarmins IL-1 α and HMGB-1 (96, 97).

Since the finding that inflammasome proteins are upregulated after TBI in human patients (98), significant attention has been paid to identify the inflammasome-associated signaling events that are engaged in response to brain trauma (14, 99, 100). Inflammasome literature has identified the expression of NLRP1, NLRP2, and NLRP3 as well as AIM2 inflammasomes in microglia, neurons, and astrocytes in the CNS (100–104). Furthermore, recent studies in CNS injury models have uncovered critical roles for inflammasome signaling in driving inflammatory responses following tissue damage in the CNS. For instance, SCI leads to the upregulation and assembly of

NLRP1 inflammasome components in spinal cord neurons (105). Moreover, neutralizing anti-ASC antibody treatment was also found to improve histopathological and functional outcome following SCI in these studies. In stroke models, methods to reduce inflammasome signaling, such as anti-NLRP1 neutralizing antibodies and caspase-1 inhibitors, as well as NLRP3, ASC, NLRC4, and AIM2 knockout mice, have all shown signs of improved functional recovery and reductions in inflammasome signaling (73, 103, 106, 107). Similarly, in an intracerebral hemorrhage model, both small interfering RNA and a selective inhibitor of the purinergic receptor P2X7R, which has been shown to promote NLRP3 activation in some experimental settings (108), limited inflammasome activation and led to neuroprotection (109). Considering the consistent benefits of inhibiting inflammasome components across these models, the inflammasome provides a tempting target for alleviating CNS injury.

Additional insights into the timing and importance of inflammasomes in CNS injury have been gained from recent TBI studies. In an FPI model, inflammasome components, such as ASC and caspase-1, were shown to be upregulated in cortical neurons for up to 24 h post-injury (110). Co-immunoprecipitation of inflammasome proteins also demonstrated that NLRP1 and ASC could be detected in multiprotein complexes in the brain. Treatment with an ASC-neutralizing antibody reduced caspase-1 activation and IL-1 β production while also decreasing lesion volume, suggesting beneficial effects of targeting inflammasome activity. Liu et al. also recently showed that TBI results in upregulated expression of NLRP3, ASC, and caspase-1. Moreover, they report that the expression of these inflammasome-associated proteins can remain elevated out to 7 days post-injury (111). Importantly, inflammasome components in this model localized not only to neurons, but also to astrocytes and microglia, suggesting a wide range of inflammasome activation across cell types. Measurements of IL-1 β and IL-18 protein levels also demonstrated that while IL-1 β peaks around 6 h after injury and subsequently decreases over time, IL-18 expression remains elevated through 7 days after injury. In agreement with these findings, a separate study also reported elevated IL-18 production for at least a week post-TBI in both humans and experimental animals (112). These data suggest that early inflammasome production of IL-1 β may be involved in acute inflammation and tissue damage, while inflammasome-driven IL-18 may contribute to the perpetuation of TBI-induced inflammation. It should be noted, however, that in a more recent study neither NLRP1 nor ASC knockout mice exhibited any improvements in lesion volume, histopathology, cell death, or motor function following CCI injury (81). It is possible that differences in the extent of caspase-1 abrogation and/or the timing of inflammasome inhibition or differences in injury models may help explain the disparate results that were reported in these studies.

Although key roles for inflammasomes have been clearly identified in other models of sterile inflammation and trauma, the specific contributions of inflammasome activation to TBI pathogenesis have only recently been investigated and multiple questions remain. For instance, although the formation of inflammasome complexes has been reported following TBI, the

roles that specific inflammasomes play in driving TBI-associated pathology and neurological dysfunction have not been studied in great detail in animal models. In addition, the individual contributions of inflammasome-derived cytokines (i.e., IL-1 α , IL-1 β , and IL-18) and caspase-1-mediated cell death in TBI pathogenesis still remain poorly characterized. The major cell types in which inflammasomes operate to promote TBI progression have also not been formally defined to date. The genetic targeting of inflammasome signaling components in mice has aided in the discovery of critical roles for inflammasomes in other models of sterile inflammation. Future *in vivo* TBI studies that utilize these genetic tools should help to more fully characterize the contributions of specific aspects of inflammasome signaling in brain trauma.

Interleukin-6

Interleukin-6 has frequently been associated with TBI outcome in humans, but it is unclear whether its role is primarily beneficial or detrimental. Microdialysis fluid detection of parenchymal IL-6 production has been associated with improved survival in TBI patients (21). However, more recent evidence points to a detrimental role for IL-6 in TBI (113). In these studies, plasma levels of IL-6 were shown to be significantly higher in severe TBI patients over moderate TBI patients. Both subacute and chronic serum levels of IL-6 have been associated with unfavorable short and long-term outcomes (75). In separating human patients by high or low cerebrospinal fluid (CSF) IL-6 trajectory, high trajectory patients are much more likely to have unfavorable clinical outcomes (76). Thus, while the role of IL-6 in TBI is still somewhat unclear, data from TBI patients indicate that IL-6 is consistently upregulated after TBI and can remain elevated in chronic stages, making it a potentially important mediator of long-term outcome.

Early animal studies verified that IL-6 is elevated in CSF and serum after TBI (114). Evidence from IL-6 knockout mice has also confirmed it as a pro-inflammatory cytokine that recruits activated glia and immune cells to sites of injury. Indeed, genetic ablation of IL-6 in cryolesioned mice resulted in fewer reactive astrocytes and macrophages and increased neuronal death (115). Conversely, overexpression of IL-6 in astrocytes enhanced recruitment of glia and immune cells to the lesion site and decreased both oxidative stress and neuronal death (115, 116). These studies suggest that IL-6's role in inducing inflammation and glial scar formation is important in reducing prolonged cell death. A later CCI study also pointed to beneficial effects of IL-6 by showing that its deficiency leads to significantly poorer performance on behavioral tests as well as higher IL-1 β protein levels in the cortex, suggesting that IL-6 may be an important regulator of IL-1 β in TBI (117). However, a more recent study using a weight drop model showed that systemic neutralization of IL-6 mitigates some of the inflammatory and behavioral effects of hypoxia on exacerbating post-injury responses, implying that reducing the inflammatory response induced by IL-6 can indeed provide neuroprotection and lead to better outcome (118). When considering these types of studies, it is important to keep in mind the difference between complete or partial removal of a gene and/or its product. It is likely that some level of IL-6 is necessary to produce an inflammatory state that positively affects outcome

such that either complete elimination or overexpression of IL-6 can be detrimental.

Tumor Necrosis Factor α

Early work on the role of tumor necrosis factor (TNF α) in TBI mouse models suggested that it has early deleterious effects after TBI while exhibiting more protective effects in chronic stages (119). However, other work suggested that TNF α is necessary to protect from early mortality within a week of injury (120). Regardless of these contradictions, literature on TNF α in TBI consistently shows an upregulation of this cytokine after injury (74, 75, 113, 121), suggesting an important role for TNF α in both the acute and chronic phases.

The importance of TNF α early after injury was recently confirmed in a weight drop model. In this study, mice receiving a TNF α inhibitor at 1 and 12 h after injury showed improved cognitive performance 1 week post-injury, but mice administered the inhibitor at 18 h post-injury did not, implying a very short window for TNF α -targeting therapeutics after TBI (122). Further investigation of the mice given the inhibitor at 1 h showed fewer apoptotic neurons and less astrogliosis at 72 h after injury in both the cortex and dentate gyrus. This study outlines a 12-h window after injury during which the detrimental effects of TNF α may be attenuated, and points toward a tentative link between TNF α and prolonged astrogliosis and neuronal death.

Aside from defining the timing of TNF α activity in TBI, it will be important to elucidate the pro-apoptotic and pro-survival pathways in which it participates following brain trauma. In conjunction with its role as a major inflammatory switch, TNF α is known to induce both cell proliferation and apoptosis through several signaling pathways. While its activation of transcription factors, such as NF- κ B and AP-1, can lead to transcription of inflammatory and apoptotic mediators, signaling through death receptors to activate caspases can also play an important part in determining cell death or survival. A recent article by Longhi et al. showed that separate deletion of either TNF receptor 1 (TNFR1) or 2 (TNFR2) can have opposite effects on cell survival and behavioral deficits (123). Using p55 (TNFR1) and p75 (TNFR2) knockout mice in a CCI model of TBI, they showed that TNFR1 deletion attenuated neuroscore deficits through 4 weeks post-injury and led to a shift to pro-survival signaling along with attenuated neuronal death and smaller lesion volume. TNFR2 knockout had the opposite effect in worsening neuroscore with no signs of pro-survival signaling or protection from cell and tissue loss. The TNFR1 knockout mice also showed a smaller area stained for CD11b as well as a higher area stained for Ym1, a marker for anti-inflammatory macrophage phenotypes, compared to the TNFR2 knockout group, suggesting opposite roles for the two receptors in recruiting inflammatory macrophages and microglia to the site of injury. These data agree with a paper by Yang et al. in which TNFR2/Fas knockout mice showed worse motor and cognitive performance after CCI TBI, although in that study neither TNFR1 or TNFR2 knockout alone had an effect on lesion volume or the number of dead cells in the cortex (124). Together, these studies indicate that the TNF receptors may play different roles post-injury, with TNFR2 providing a neuroprotective role and TNFR1 playing a detrimental one.

An important consideration about TNF α signaling is that due to the much wider expression of TNFR1 across cell types as well as its ability to respond to both forms of TNF (both soluble and transmembrane), this receptor could have more potent inflammatory consequences than its counterpart TNFR2. In addition, it has been shown that TNFR1 can signal through NF- κ B, JNK, and caspase-mediated apoptotic pathways, while it is more common for TNFR2 to engage NF- κ B and PI3K to induce pro-inflammatory and pro-survival signaling (125, 126). Thus, consolidating seemingly contradictory evidence for the role of TNF α in TBI with regards to cell death and clinical outcome will likely involve understanding the conditions under which its various forms and receptors participate in different survival or death pathways and the timeline on which this signaling can occur.

Granulocyte Colony-Stimulating Factor/ Granulocyte Macrophage Colony- Stimulating Factor

Both granulocyte colony-stimulating factor (G-CSF) and GM-CSF are involved in the expansion and mobilization of immune cells from the bone marrow and act as key cytokines in the inflammatory response. Interestingly, some recent evidence suggests that both G-CSF and GM-CSF may play a protective role in TBI. A recent paper using G-CSF administration after a CCI injury showed that G-CSF injection improves cognitive recovery and increases neurogenesis in the hippocampus (127). This was accompanied by higher activation of astrocytes and microglia as well as higher levels of brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF), indicating that G-CSF may regulate production of neurotrophic factors by activated glia post-TBI to promote neurogenesis. Similarly, a study using GM-CSF knockout mice showed that GM-CSF deficiency in TBI results in more cognitive deficits with higher tissue and neuronal loss after FPI (128). GM-CSF knockout mice also showed reductions in astrogliosis, which may suggest that GM-CSF plays a role in activating astrocytes to protect cells and boost tissue repair. Understanding how both of these molecules interact with glia to promote neuronal protection and regeneration may elucidate how other neuroprotective processes may involve glial functions.

Type 1 Interferon

Increasing evidence points toward central roles of type 1 interferon (IFN) signaling in inflammatory CNS disorders and age-related cognitive decline (129–132). A recent study by Karve et al. is one of the first to investigate the involvement of type 1 IFN signaling in TBI pathogenesis (82). They found that deficiency in type 1 IFN signaling produced by either type 1 IFN receptor (IFNAR) knockout or an IFNAR blocking antibody reduces lesion volume. This neuroprotection was associated with a shift toward more anti-inflammatory cytokine signaling; however, this also coincided with increased GFAP and IBA1 staining. In addition, using a bone marrow chimera, they found that IFNAR deficiency in hematopoietic cells alone was sufficient to confer lesion volume protection and elevated GFAP and IBA1 staining. Importantly, they also showed that brain trauma in humans

promotes enhanced expression of type-1 IFN, which suggests that type-1 IFN signaling may potentially influence clinical outcome in TBI patients.

Interleukin-10

Interleukin-10 has been shown to be elevated in TBI patients (78, 133–135) and has been associated with unfavorable outcome and mortality (75, 134, 135). Despite these associations, Chen et al. found a role for IL-10 in conferring neuroprotection with hyperbaric oxygen (HBO) treatment (80). They found that the protective effects of HBO in TBI included reduced lesion volume and edema, improvements in cognitive performance, and the dampening of pro-inflammatory cytokine production in the cortex. It also led to a shift from apoptotic to cell survival pathways and greater BBB integrity. This wide range of positive effects was diminished in IL-10-knockout mice, and IL-10 injection by itself improved lesion volume, edema, and cognitive outcome in both wild-type and IL-10-knockout animals. This indicates an important protective role for IL-10 in TBI as well as a way to induce its production through HBO treatment. It is possible that the association between IL-10 (and likely many other cytokines) and poor outcome is primarily due to a widespread upregulation of cytokines after TBI, and that a more informative approach to understanding the role of IL-10 after brain injury involves dissecting its specific roles in damage responses.

Transforming Growth Factor β

Transforming growth factor β (TGF β) increases acutely in the serum and CSF of TBI patients (136). Several mediators of TGF β signaling have been shown to be upregulated in TBI models (79, 137, 138). For example, transforming growth factor beta-activated kinase 1 (TAK1) was shown to increase in expression and is detected in cortical neurons and astrocytes after weight drop TBI (79). Inhibition of TAK1 signaling improved neuronal survival and motor function and also decreased NF- κ B activity and inflammatory cytokine release. Transforming growth-interacting factor (TGIF), a transcriptional co-repressor of TGF β that can inhibit transcriptional activation of TGF β , was demonstrated to be upregulated in TBI animals and localized to both neurons and microglia (138). Using small hairpin RNA to knockdown TGIF levels in the brain, the authors found that lower TGIF levels led to a decrease in infarct volume and microglia number around the lesion, as well as a change in microglia morphology. Knockdown of TGIF also improved motor function through 2 weeks after injury. These data indicate that mediators of TGF β signaling can have important inflammatory consequences.

FUTURE PERSPECTIVES

As highlighted in this review, increasing experimental evidence indicates that the immune system can profoundly influence clinical outcomes following TBI. Importantly, various recent studies have shown that targeting immune signaling with genetic and pharmacological approaches can lead to significant improvements in neurological function and tissue repair post-TBI. Both neuroprotective and detrimental roles have been assigned to the immune system in TBI. Whether the immune response

contributes to beneficial tissue repair or further brain damage largely depends on the nature, kinetics, and magnitude of the inflammatory response. Although targeting the immune system has emerged as an exciting potential strategy to treat TBI, there are numerous outstanding questions that need to be addressed to better characterize the involvement of immune signaling in TBI etiology and to realize the full potential of immune-based therapeutics.

For one, there is still an overall lack of consensus on the overarching roles that discrete immune cell types and pathways play in TBI. Future efforts are needed to help reconcile the biological reasons that account for the disparate results that have been reported on immune mediators in TBI by different labs. Much of the inconsistency in the literature can be attributed to the utilization of different approaches to induce brain trauma and modulate immune signaling between labs (Table 2). Pinpointing

TABLE 2 | Genetic models used to characterize the role of immune cell types and signaling pathways in TBI.

Cell type	Animal line/model	Purpose	Major findings in TBI animals	Reference
Neutrophils	IgM RP-3	Neutrophil depletion	No significant decrease in BBB permeability	(36)
	Anti-Gr1 antibody	Neutrophil depletion	Decreased edema, apoptosis, and microglia/macrophage activation, no significant changes in BBB integrity	(38)
	CXCR2 knockout	Reduce neutrophil infiltration	Decreased cell death, no significant changes in BBB permeability or behavior	(39)
	Neutrophil elastase knockout	Reduce neutrophil effector functions	Decreased edema and apoptotic neurons, but no decrease in tissue volume loss or behavioral improvement	(40)
Macrophages and microglia	CD11b-TK	Deplete CD11b-expressing cells	Reductions in microglia numbers in the brain, no improvement in axonal injury, treatment toxic at high dosage	(45)
	CD11b-DTR	Deplete CD11b-expressing cells	No change in lesion size, treatment caused inflammatory response without injury	(46)
	CCX872 (CCR2 antagonist)	Reduce CCR2 signaling functions	Reduced macrophages in the brain, altered pro- and anti-inflammatory cytokine expression, less cognitive dysfunction	(47)
	CCR2 knockout	Limit CCR2-mediated recruitment of monocytes	Reduced numbers of infiltrating monocytes, improved learning and memory	(48)
	CCR2 ^{RFP/RFP}	Disrupt recruitment of monocytes	Reduced monocyte recruitment, cavity volume, and axonal pathology	(49)
	CX3CR1 knockout	Abrogate CX3CR1 signaling functions in macrophages and microglia	Short-term neuroprotection and lower inflammatory response, long-term functional impairments and elevated myeloid cell activation	(50)
T cells	Rag1 knockout	Genetic ablation of B and T cells	No changes in neurological outcome, BBB integrity, pro- or anti-apoptotic mediators, hippocampal architecture, or astroglial activation	(58)
	FTY720	Sequester lymphocytes and reduce their migration to the brain	Decreased circulating lymphocytes, decreased neutrophils and macrophages/microglia in ipsilateral hemisphere	(59)
Inflammatory mediator	Animal line/model	Purpose	Major findings in TBI animals	Reference
IL-1	Anti-IL-1 β antibody	Blockade of IL-1 β signaling	Reductions in macrophages/microglia, neutrophils, and T cell numbers in the brain, improvement in learning tasks, and decreased tissue loss	(87, 88)
	IL-1R antagonist	Neutralize IL-1	Higher expression of proinflammatory cytokines in macrophages	(93)
ASC	Anti-ASC	Limit inflammasome assembly	Reduced caspase-1 activation and IL-1 β production, decreased lesion volume	(110)
	ASC knockout	Abrogate inflammasome assembly	No improvements in lesion volume, histopathology, cell death, or motor function	(81)
NLRP1	NLRP1 knockout	Prevent NLRP1 inflammasome assembly	No improvements in lesion volume, histopathology, cell death, or motor function	(81)
IL-6	IL-6 knockout	Ablation of IL-6 signaling	Fewer reactive astrocytes and macrophages, increased neuronal death	(115)
	IL-6 knockout	Ablation of IL-6 signaling	Poor behavioral performance, higher IL-1 β levels in the cortex	(117)
	GFAP-IL-6 overexpression	Increase IL-6 expression in astrocytes	Greater recruitment of glia and immune cells to the lesion, decreased oxidative stress and neuronal death	(116)
	Anti-IL-6 antibody	Neutralize IL-6	Reduced some inflammatory and behavioral effects of post-injury hypoxia	(118)
TNF α	TNF α inhibitor post-TBI	Inhibit TNF α signaling	Early administration improved cognitive performance, and decreased neuronal apoptosis and astrogliosis	(122)
	TNFR1 knockout	Disrupt TNF α signaling through TNFR1	Improved neurological function and neuronal survival/lesion volume, decreased numbers of CD11b ⁺ cells in the brain	(123)
	TNFR2 knockout	Reduce TNFR2 signaling	Worsened neurological function and no protection from tissue loss	(123)

(Continued)

TABLE 2 | Continued

Inflammatory mediator	Animal line/model	Purpose	Major findings in TBI animals	Reference
	TNFR2/Fas knockout	Abrogate TNF α signaling through TNFR2	Impaired motor and cognitive performance	(124)
G-CSF	G-CSF injection post-TBI	Enhance G-CSF signaling	Improved cognitive performance and increased hippocampal neurogenesis, higher glial activation and production of BDNF and GDNF	(127)
GM-CSF	GM-CSF knockout	Disrupt GM-CSF signaling	Worsened cognitive deficits as well as cell and tissue loss, reduced astrogliosis	(128)
Type 1 IFN	IFNAR knockout or IFNAR blocking antibody	Block type 1 IFN signaling	Reduced lesion volume, more anti-inflammatory cytokine signaling, increased glial activation, these effects were hematopoietic cell-dependent	(82)
IL-10	IL-10 knockout, IL-10 injection	Modulate IL-10 signaling	Diminished protective effects of hyperbaric oxygen treatment, including lesion volume, edema, cognitive improvement, and decreased cytokine production in IL-10 knockout mice, while IL-10 injection improved these outcomes	(80)
TGF- β	TAK1 inhibition	Disrupt signaling downstream of TGF- β	Improved neuronal survival and motor function, decreased NF- κ B signaling and inflammatory cytokine production	(79)
	TGIF shRNA knockdown	Ablation of downstream TGF- β signaling	Decreased infarct volume and microglia numbers, improved motor function	(138)
APOE	APOE ϵ 4 overexpression	APOE ϵ 4 overexpression	Worsened brain pathology, BBB breakdown, and neurological impairments	(156, 157)
TREM2	TREM2 knockout	Abrogate TREM2 signaling	Altered macrophage distribution, hippocampal neuroprotection, and fewer cognitive deficits	(83)

what is mechanistically responsible for the conflicting findings in the TBI literature will help to uncover the important nuances of the immune response to brain trauma and will aid in the identification of optimal therapeutic regimens to treat discrete types of CNS injury.

One thing that is largely agreed upon in the literature is that no two brain injuries are alike and that seemingly similar types of trauma can result in diverse clinical outcomes. Unfortunately, the reasons for the heterogeneity in disease sequelae and recovery time associated with comparable forms of trauma remain poorly understood. Heterogeneity in recovery time and extent of neurological dysfunction in the TBI patient population can only be partially explained by differences in brain injury severity and location. This has led to greater appreciation for the roles of host genetics, environmental factors, lifestyle choices, and previous TBI history in overall clinical outcome. It is currently difficult to predict how interplay between these diverse non-injury factors affect TBI pathogenesis, and future studies are greatly needed to uncover their influence on TBI. Unfortunately, current TBI treatment approaches do not fully take into consideration many of the non-injury factors that are described above. The utilization of immune-based biomarkers in the future may offer a strategy to improve the stratification and treatment of TBI patient groups. Recent advancements from experimental TBI models indicate that the nature and kinetics of the immune responses can vary depending on brain injury location and severity. Furthermore, immune responses are also significantly affected by environmental and lifestyle factors (e.g., diet, antibiotics usage, prescription, or recreational drug use), microbiome composition, individual

genetic factors, and previous TBI history. Therefore, it is feasible that immune cell frequencies and cytokine production in patients may serve as valuable biomarkers to predict clinical outcomes, stratify patient groups, and to maximize therapeutic approaches to treat TBI.

Mounting epidemiological evidence indicates that TBI is a major risk factor for developing numerous neurological disorders including AD, ALS, CTE, and posttraumatic stress disorder (PTSD) (5–10, 139, 140) and also possibly MS (141–143). Although it is widely appreciated that TBI predisposes individuals to other neurological disorders, how TBI mechanistically contributes to CNS disease later in life still remains poorly understood. Dysregulated immune responses have been identified to play key roles in the pathogenesis of the majority of neurological disorders and mental illnesses that have been linked to TBI. As a result, it is tempting to speculate that the aberrant inflammatory conditions that are generated in response to brain trauma may set in motion a series of events that can contribute to the development of other neurological disorders over time. Indeed, recent advances have been made in characterizing how TBI can contribute to AD pathogenesis. These studies have shown that brain trauma can spur the aberrant release and deposition of both amyloid beta (A β) and tau (144). The accumulation of neurotoxic forms of A β and tau are believed to be major drivers of AD pathogenesis (145), and studies of postmortem brains following brain trauma indicate that A β and tau deposition are also hallmarks of TBI (144, 146–152). The mechanism(s) by which A β and tau promote AD is still a matter of great debate; however, emerging data clearly point to roles for A β - and tau-induced neuroinflammation in

this process (153). Therefore, it is conceivable that neuroinflammation and neuronal damage that is incited by the deposition of A β and tau following TBI may instigate a pathological cycle of continued A β and tau release and inflammation that initiates early AD progression. Furthermore, targeting the hyperinflammatory responses that are generated in response to A β and tau accumulation post-brain trauma could help to limit the risk of AD development in TBI patients (154, 155).

Interest in the link between TBI and AD has also extended to some of the major genetic susceptibility factors that are associated with AD. Genome-wide association studies (GWAS) have determined that carrying an allele of APOE ϵ 4 (apolipoprotein E ϵ 4) or a mutation in TREM2 (triggering receptor expressed on myeloid cells 2) is associated with significantly higher rates of AD in humans (156). Interestingly, recent reports suggest that manipulation of either TREM2 or APOE biology can also affect the severity of brain pathology and neurological dysfunction following TBI (83, 157). For instance, it was shown that TBI results in more severe memory and functional impairments in individuals who carry the APOE ϵ 4 allele than in people who possess other APOE alleles (154, 158). Moreover, transgenic overexpression of APOE ϵ 4 in mice was also found to cause worsened brain pathology, BBB breakdown, and neurological impairments following brain injury (159, 160).

TREM2 is an immunoglobulin-superfamily receptor that is predominantly expressed on myeloid cells including microglia, macrophages, and osteoblasts. Recent studies have shown that

TREM2 is involved in the removal of debris, misfolded proteins, and phospholipids from the CNS (161). An important feature of both AD and TBI is that A β can accumulate into plaques (157), which are thought to lead to detrimental effects on neurological function if not cleared by phagocytes. TREM2 can help microglia and infiltrating macrophages detect lipoprotein-bound A β in the brain and trigger phagocytosis (162) and may also participate in maintaining the survival of these cells (163) (Figure 3). These activated cells can then recruit more phagocytes to the sites of A β accumulation. Downstream effects of this phagocytic response remain unclear, but multiple studies have agreed that TREM2 deficiency in various CNS disorders does lead to a decreased number of phagocytes, which likely impairs debris clearance (83, 164–166). In such cases of TREM2 mutation or dysfunction, it is possible that A β and other misfolded proteins may not only be insufficiently cleared, but may also accumulate faster. A recent article by Saber et al. used an FPI injury model to explore the effect of TREM2 on TBI-induced neuroinflammation, tissue loss, and neurological function (83). They found that TREM2-knockout mice did indeed show fewer macrophages throughout the brain, but more were present close to the site of injury. This was associated with hippocampal neuroprotection and fewer cognitive deficits in the TREM2 knockouts. Interestingly, sham TREM2-knockout mice appeared to have some differences from sham wild-type mice in certain behaviors. This paper shows that TREM2 is also likely to be important in TBI recovery; thus, it will be interesting going

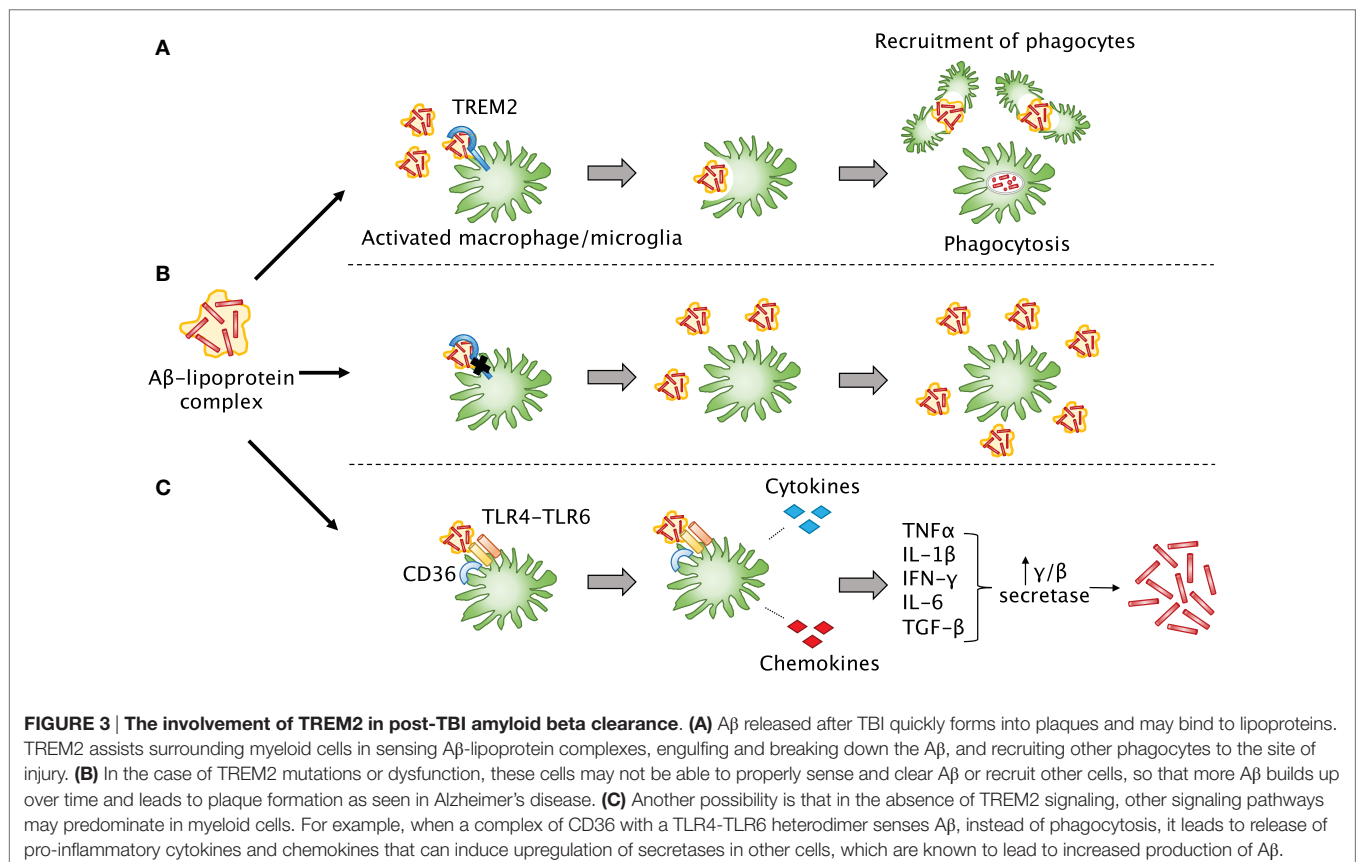


FIGURE 3 | The involvement of TREM2 in post-TBI amyloid beta clearance. (A) A β released after TBI quickly forms into plaques and may bind to lipoproteins. TREM2 assists surrounding myeloid cells in sensing A β -lipoprotein complexes, engulfing and breaking down the A β , and recruiting other phagocytes to the site of injury. **(B)** In the case of TREM2 mutations or dysfunction, these cells may not be able to properly sense and clear A β or recruit other cells, so that more A β builds up over time and leads to plaque formation as seen in Alzheimer's disease. **(C)** Another possibility is that in the absence of TREM2 signaling, other signaling pathways may predominate in myeloid cells. For example, when a complex of CD36 with a TLR4-TLR6 heterodimer senses A β , instead of phagocytosis, it leads to release of pro-inflammatory cytokines and chemokines that can induce upregulation of secretases in other cells, which are known to lead to increased production of A β .

forward to elucidate the mechanisms by which it mediates debris clearance and neuroinflammation.

Despite the recent progress that has been made in characterizing how having a history of TBI can predispose individuals to AD, little is currently known about what biologically accounts for the increased risk of developing other neurological conditions in TBI patients. Immune dysfunction has been implicated in the pathogenesis of many of these CNS disorders that are more prevalent following brain trauma, including CTE, mental illness, and PTSD (167–169). Therefore, targeting the immune system following TBI may help to lower the risk of developing other neurological diseases later in life.

CONCLUSION

Once considered a silent epidemic, TBI is now recognized as a serious threat to global human health. In recent years, considerable efforts and resources have been paid to reduce the rates and severity of TBI. Unfortunately, these preventative measures have been largely unsuccessful, and the number of individuals who sustain debilitating brain trauma each year continues to rise. As highlighted in this review, recent advancements in animal models of TBI clearly indicate that immune responses are centrally involved in the development of brain pathology and neurological dysfunction following TBI. Importantly, these emerging studies

suggest that targeting the immune system could offer a much-needed therapeutic approach to treat TBI. Future studies that are geared toward further defining the major immunological pathways that influence TBI pathogenesis will lead to an improved understanding of brain injury etiology and will aid in the identification of novel immune-based TBI treatment strategies.

AUTHOR CONTRIBUTIONS

CM wrote the sections of the manuscript related to the involvement of immune cell types and inflammatory mediators in TBI; designed the manuscript; and created the figures. JL wrote the introduction, future perspectives, conclusion, and sections of the manuscript related to inflammasomes and the kinetics of the immune response to brain injury; designed the manuscript; and oversaw the entire process of manuscript preparation.

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Bone Marrow-Derived Cell Accumulation in the Spinal Cord Is Independent of Peripheral Mobilization in a Mouse Model of Amyotrophic Lateral Sclerosis

Kyle Peake¹, John Manning¹, Coral-Ann Lewis², Kevin Tran¹, Fabio Rossi² and Charles Krieger^{1,3*}

¹ Department of Biomedical Physiology and Kinesiology, Simon Fraser University, Burnaby, BC, Canada, ² The Biomedical Research Centre, University of British Columbia, Vancouver, BC, Canada, ³ Division of Neurology, Department of Medicine, Neuromuscular Disease Unit, VHHSC, Vancouver, BC, Canada

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Luc Dupuis,
INSERM U1118, France

*Correspondence:

Charles Krieger
ckrieger@sfu.ca

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Bone marrow-derived cells (BMDCs) are capable of migrating across the blood–brain barrier (BBB) and accumulating in the central nervous system (CNS) when transplanted into recipients conditioned with whole-body irradiation or chemotherapy. We used the chemotherapeutic agents busulfan and treosulfan to condition recipient mice for transplantation with bone marrow (BM) cells isolated from donor mice ubiquitously expressing green fluorescent protein. We attempted to increase the accumulation of BMDCs in the CNS by mobilization of BMDCs using either, or both, granulocyte colony-stimulating factor (G-CSF) or plerixafor (AMD3100). We also used several concentrations of busulfan. We hypothesized that higher concentrations of busulfan and BMDC mobilization would increase numbers of GFP⁺ cells in the CNS. The doses of busulfan employed (60–125 mg/kg) all resulted in high levels of sustained chimerism (>85% 1 year post-transplant) in both the blood and BM of wild-type (WT) mice and an amyotrophic lateral sclerosis (ALS) mouse model. Moreover, cells accumulated within the CNS in a dose-, time-, and disease-dependent manner. Conditioning with the hydrophilic busulfan analog treosulfan, which is unable to cross the BBB efficiently, also resulted in a high degree of BM chimerism. However, few GFP⁺ BMDCs were found within the CNS of WT or ALS mice of treosulfan-conditioned mice. Mobilization of BMDCs into the circulation using G-CSF and/or AMD3100 did not lead to increased accumulation of GFP⁺ BMDCs within the CNS of WT or ALS mice. Weekly analysis of BMDC accumulation revealed that BMDCs accumulated more rapidly and to a greater extent in the CNS of ALS mice conditioned with a high dose (125 mg/kg) of busulfan compared to a lower dose (80 mg/kg). The number of GFP⁺ BMDCs in the CNS labeling with the proliferation marker Ki67 increased in parallel with BMDC accumulation within the CNS. Our results indicate that establishment of high levels of blood and BM chimerism alone is not sufficient to induce BMDC accumulation within the CNS and that CNS conditioning is a crucial requirement

for BMDC accumulation to occur. Moreover, it appears that proliferation of BMDCs that infiltrate the CNS is partly responsible for cell accumulation in busulfan-conditioned ALS mice.

Keywords: busulfan, treosulfan, amyotrophic lateral sclerosis, bone marrow-derived cells, monocyte, granulocyte colony-stimulating factor, AMD3100, central nervous system

INTRODUCTION

Following bone marrow (BM) transplantation (BMT), donor BM-derived cells (BMDCs) can migrate to various sites in the recipient including the BM and the central nervous system (CNS) (1). It is generally believed that the BMDCs that accumulate within the CNS are predominantly of monocytic lineage (2–4). These BM-derived monocytic lineage cells integrate within the CNS, exhibit several microglial markers, and have morphologies reminiscent of endogenous microglia and perivascular macrophages associated with blood vessels (2). Recent studies have shown that some populations of macrophages at CNS interfaces, such as perivascular macrophages, have stable populations and do not necessarily derive from BMDCs (5).

For successful BMT, niche space must be generated within the recipient BM compartment to allow for donor cell engraftment. While this is most commonly achieved using myeloablative irradiation, this procedure can lead to lethal damage and inflammation in the CNS as well as immunosuppression that increases the potential for secondary infections (6). Consequently, a number of recent reports have explored the utility of non-irradiative conditioning protocols. Busulfan (BU) is a clinically approved bifunctional alkylating agent that depletes non-cycling primitive stem cells in the BM and has weak immunosuppressive properties (7). We and others have found that conditioning with BU prior to BMT leads to high levels of BM chimerism and accumulation of BMDCs within the CNS (2–4, 8–12). In fact, Wilkinson et al. have claimed that BU conditioning enhances engraftment of BMDCs compared to whole-body lethal irradiation when using a higher dose of BU (125 mg/kg), than we and others have used previously (4).

The microglial population is established by primitive myeloid cells during embryogenesis and is largely self-sustaining in the adult CNS (13). In parabiotic models where chimerism is established physiologically by surgically connecting the circulations of two mice, rather than by injecting whole BM into the circulation, accumulation of donor BMDCs in the CNS does not occur even after irradiation of the parabiotic recipient (14). Similarly, transplantation of BMDCs following whole-body irradiation does not lead to accumulation of BMDCs in the CNS if the brain is protected by shielding, demonstrating the importance of brain conditioning for BMDC accumulation to occur in the CNS (15). Together, these observations suggest that reconstitution of the CNS with BMDCs requires both the presence of monocytic lineage progenitors in the blood circulation capable of efficient transmigration across the blood–brain barrier (BBB), and conditioning of the BBB/CNS, possibly through ablation of endogenous proliferating microglia (8).

Given the potential utility of BMDCs as a vehicle to deliver therapeutics to the CNS, we sought to further elucidate the mechanisms involved in BMDC accumulation within the CNS of a mouse model of amyotrophic lateral sclerosis (ALS). ALS is a neurodegenerative disease characterized by the progressive loss of motoneurons in the brainstem and spinal cord, as well as neuron loss in the cerebrum. ALS pathogenesis is heterogeneous and is associated with mutations in several genes, as well as with other pathological processes including excitotoxicity, oxidative injury, protein aggregation, and altered RNA metabolism that result in cellular dysfunction (16). The gene mutations found in ALS include those for Cu/Zn superoxide dismutase (SOD1), which subsequently led to the development of transgenic mice over-expressing human mutant SOD1 (mSOD) as a murine model of ALS (17). The G93A mSOD transgenic model develops progressive motoneuron degeneration and limb paralysis mimicking human ALS.

Regardless of the initiating causes of ALS, pathologically ALS is characterized by activation and proliferation of microglia, in addition to the neuron loss, suggesting that microglia contribute to ALS pathogenesis (16). Recent work has claimed that the increased number of monocyte lineage cells found in the spinal cords of mSOD mice, and possibly ALS patients, is due to recruitment of circulating Ly6C^{hi} monocytes (18). Butovsky and colleagues reported that recruited Ly6C^{hi} monocytes were detected in the spinal cord early in disease and that the number of Ly6C^{hi} cells increased as disease progressed, reflecting further recruitment of monocytes. By contrast, the population of resident microglia, identified using the marker CD39, decreased with disease progression (18). Use of anti-Ly6C monoclonal antibody against infiltrating monocytes slowed disease progression, presumably by decreasing the entry of recruited Ly6C monocytes to the spinal cord (18). However, using RNA sequencing and evaluation of specific expression patterns of spinal cord microglia and peripheral monocytes, Chiu et al. found resident microglia increased in number during disease progression, but that the monocyte population did not increase, unlike the results of Butovsky et al. (19). Furthermore, Chiu et al. did not observe many Ly6C^{hi} cells in spinal cord, suggesting limited infiltration of peripheral monocytes (19). In previous studies where we generated parabiotic mice pairs using wild-type (WT) mice ubiquitously expressing green fluorescent protein (GFP) and mSOD mice, we did not observe any entry of GFP⁺ cells from the parabiont into the CNS of the mSOD mouse, suggesting that there is no infiltration of monocytes into the CNS of mSOD mice under physiological conditions (14). Notably, parabiosis cannot be maintained when the mSOD mouse becomes very debilitated, limiting the evaluation of peripheral monocyte recruitment in late disease stages.

In the present study, we transplanted donor BM cells isolated from mice ubiquitously expressing GFP in order to monitor and characterize BMDC accumulation in BU-conditioned mice. Using this transplantation model, we evaluated the effects that different doses of BU had on BMDC accumulation within the CNS. The current literature suggests that both circulating progenitors and CNS conditioning are required for BMDC accumulation within the CNS. As such, we also monitored the effects of pharmacological mobilization of BMDCs, and the consequences of minimizing CNS conditioning by using the BU analog treosulfan (TREO), on BMDC accumulation within the CNS. We had three hypotheses; we expected that BMDC accumulation would be dependent on the conditioning method and that BMDC accumulation in the spinal cord would increase with time, consequent to the proliferation of the BMDC within the CNS. We also hypothesized that mobilization of BMDC from BM would increase the number of monocyte lineage cells entering the spinal cord and contributing to BMDC accumulation.

MATERIALS AND METHODS

Ethics Statement

All protocols related to the use of animals in this study were reviewed and approved by the University Animal Care Committee of Simon Fraser University and were in compliance with the Canadian Council of Animal Care, the NIH Guide for the Care and Use of Laboratory Animals, and the EEC Council Directive.

Animals

A colony of transgenic mice that over-express mutant human superoxide dismutase-1 (mSOD; B6.Cg-Tg(SOD1*G93A)1Gur/J; Jax 004435) was established at Simon Fraser University using breeding pairs obtained from Jackson Laboratories (Bar Harbor, ME, USA). The mice were maintained under temperature-controlled conditions with a 12-h light:12-h dark cycle, and were supplied with food and water *ad libitum*. As mSOD⁺ females are unable to successfully breed, WT females (C57BL/6J; Jax 000664) were paired with mSOD⁺ males. Genotypes of mice were determined by PCR analysis of genomic DNA isolated from ear tissue collected during notching as described previously (17). The mSOD mice develop progressive motoneuron degeneration, culminating in muscle atrophy and eventually hind limb paralysis. Unless otherwise stated, mSOD mice, as well as age- and sex-matched controls, were collected at advanced stage of disease progression defined as >5 s to right from lateral recumbency.

Donor mice ubiquitously expressing GFP under the control of the β -actin promoter (C57BL/6; GFP/CD45.2) were obtained from Dr. I. Weissmann. Mice were bred and maintained as heterozygotes at Simon Fraser University, with genotypes being confirmed by observing GFP expression in collected ear tissue by fluorescence microscopy.

Myelosuppressive Conditioning

The myelosuppressive alkylating agent busulfan (Busulfex, Otsuka Pharmaceuticals, Japan) was diluted from pharmaceutical stock solution to 3 mg/mL with sterile PBS just prior to

administration. Fractionated doses of 20 mg/kg/day of BU were administered *via* intraperitoneal injections until a total dose of 60–100 mg/kg was delivered as we have done previously (3, 11, 12). For a total dose of 125 mg/kg, fractionated doses of 25 mg/kg/day were administered for 5 days (4). BMTs were conducted 24 h following the final injection of BU.

Treosulfan (TREO; Medac, DE), a hydrophilic analog of BU that does not readily cross the BBB (20, 21), was resuspended in sterile ddH₂O at a concentration of 50 mg/mL just prior to administration. Doses were selected based upon previous studies by Van Pel et al. and Nasa et al. (22, 23). Mice conditioned with 4,500 mg/kg TREO received daily intraperitoneal injections of 1,500 mg/kg/day over the course of 3 days while mice conditioned with 6,000 mg/kg TREO received daily intraperitoneal injections of 2,000 mg/kg/day over the course of 3 days. BMTs were conducted 72 h following the final injection of TREO as evidence suggests this improves grafting efficiency (23).

Bone Marrow Transplantation

Bone marrow cells were isolated from the femurs and tibiae of GFP⁺ donor mice. Briefly, donors were euthanized, and the femurs/tibiae were removed and cleaned of tissue. The end caps were shaved off the bones, and BM cells were isolated by flushing the medullary cavity with sterile PBS. Red blood cells were lysed with ACK lysing buffer (A10492-01, Life Technologies), and the BM cells were resuspended in sterile PBS at a final concentration of 5×10^6 cells/mL. Three hundred microliters of cell suspension was injected *via* the tail vein into conditioned recipients. Details of the BMT procedure are described elsewhere (11).

Measuring Extent of Chimerism

In order to estimate the extent of BM chimerism, blood was sampled from the saphenous vein and red blood cells were lysed with ACK lysing buffer. Myeloid cells were labeled with anti-CD11b-APC (1:400; 17-0112-81, eBioscience) and anti-GR1-APC (1:400; 553129, BD Pharmingen) antibodies, while lymphoid cells were labeled with anti-CD3e-PECy7 (1:200; 25-0031-81, eBioscience) and anti-CD45R-PECy7 (1:200; 25-0452-81, eBioscience) antibodies. Cells were quantified by flow cytometry using a Guava flow cytometer (EMD Millipore, DE, USA) and analyzed using FlowJo (FlowJo, Ashland, OR, USA).

During tissue collection, a single femur was removed following perfusion with PBS and prior to fixation with paraformaldehyde. BM cells were flushed from the femur using FACS buffer (2mM EDTA + 2% fetal bovine serum in PBS) and subsequently processed in a similar fashion as blood samples above.

Mobilization Experiments

Bone marrow cells were mobilized into the circulation using either granulocyte colony-stimulating factor (GCSF), AMD3100 (plerixafor), or a combination of GCSF and AMD3100. For GCSF-mediated mobilization, GCSF [Neupogen (filgrastim), Amgen, Thousand Oaks, CA, USA] was diluted from the pharmaceutical stock to 30 μ g/mL with sterile PBS + 0.1% BSA, and mice received daily intraperitoneal injections of 300 μ g/kg GCSF for five consecutive days (24). For AMD3100-mediated mobilization, AMD3100 octahydrochloride hydrate (A5602, Sigma) was

resuspended to 0.5 mg/mL with sterile PBS, and mice received a single intraperitoneal injection of 5 mg/kg AMD3100 (25, 26). To combine mobilization treatments, mice received daily intraperitoneal injections of 300 µg/kg G-CSF for 4 days followed by a single intraperitoneal injection of 5 mg/kg AMD3100 on day 5 (25, 26).

Tissue Collection and Processing

Tissue was collected as previously described (27). Briefly, mice were euthanized and transcardially perfused with 30 mL of PBS. The femur was removed to determine chimerism, and subsequently, the mice were perfused with 30 mL of 4% paraformaldehyde (w/v). Spinal columns were dissected and post-fixed in 4% paraformaldehyde at 4°C overnight. The spinal cord was removed from the spinal column, and the tissue was then cryoprotected in 20% sucrose (w/v) for 24–72 h at 4°C. Tissue was then embedded in TissueTek OCT embedding compound (Sakura Finetek, USA), wrapped in parafilm, and stored at –80°C. Tissue was processed for immunohistochemistry as described previously (27). Briefly, 30 µm sections of lumbar region of the spinal cord were sectioned on a cryostat and every fifth section was collected, with at least 10 sections per sample. Samples were stored in DeOlmos solution at –20°C until immunohistochemical staining was performed.

Assessment of Cell Proliferation

The cell proliferation-associated protein Ki67 was detected with anti-Ki67 antibody (1:1,000; ab15580, Abcam) using the free-floating immunohistochemistry procedure (2). Sections were mounted on slides using Vectashield mounting medium (Vector Labs, Burlingame, CA, USA), and proliferating cells were quantified in three individual lumbar spinal cord sections separated by at least 150 µm using a Leica epifluorescence microscope. EdU (5-ethynyl-2'-deoxyuridine; A10044, Thermo Fisher Scientific) was resuspended at a concentration of 5 mg/mL and administered to mice *via* intraperitoneal injections of 50 mg/kg EdU 48 and 24 h prior to tissue collection. Incorporation of EdU into proliferating cells was quantified using the Click-iT EdU Imaging Kit (C10640, Thermo Fisher Scientific) according to the manufacturer's instructions. Iba1 (1:1,000, 019-19741, Wako) was labeled using the free-floating immunohistochemistry procedure (2), and Iba1⁺ cells were quantified in one half of three individual lumbar spinal cord sections separated by at least 150 µm using a Leica epifluorescence microscope.

BMDC Quantification and Morphological Analysis

Spinal cord sections were mounted on slides using Vectashield mounting medium and analyzed using a Leica epifluorescence microscope. GFP⁺ cells were quantified in five individual lumbar spinal cord sections separated by at least 150 µm as we have done previously (2, 3, 12, 27). The morphology of the GFP⁺ cells was classified according to Vallières and Sawchenko (28). Briefly, “round cells” were round in shape with a diameter <9 µm; “rod cells” were oblong in shape with rounded ends and a length of ~20 µm; “elongated cells” had a length >20 µm without rounded

ends; “ameboid” cells had a variable shape with a diameter >9 µm; and “stellate cells” had a small cell body and the presence of multiple ramified processes.

Statistical Methods

All of the statistical analysis related to cell proliferation were carried out using SAS statistical software (version 9.4; SAS Canada, Toronto, ON, Canada). Analysis of GFP⁺ cell numbers and count data was used to compare the mean responses between treatments over time. The effects of treatment, time, and their two-way interaction were considered to be fixed effects in the model. *Post hoc* tests using the Tukey–Kramer method were used to locate differences in mean responses between pairs of treatments and/or timepoints. All of the model diagnostics relating to the residuals were verified. In cases where the residuals failed to satisfy the model assumptions, a natural logarithm transformation was used. Statistical analyses of cell accumulation data were conducted using SPSS software.

RESULTS

BMDCs Accumulate within the Lumbar Spinal Cord of BU-Conditioned Mice in a Time-, Dose-, and Disease-Dependent Manner

We have previously shown that myelosuppressive conditioning with 60–100 mg/kg BU, followed by BMT, was sufficient to generate mice with >80% BM chimerism after 11 weeks post-transplant (3). When mice are conditioned with 125 mg/kg BU, chimerism was rapidly established during the first few weeks post-BMT and a high degree of chimerism is maintained over the subsequent weeks in the blood (**Figure 1A**). The high level of chimerism achieved was stable, being maintained in the blood and BM of BU-conditioned WT mice for at least 1 year post-BMT (**Figure 1B**). Notably, there were no differences in the levels of chimerism achieved in the blood and BM with any of the concentrations of BU used to condition the mice (**Figure 1B**).

Conditioning with 60–100 mg/kg BU allowed for accumulation of transplanted BMDCs within the lumbar spinal cord of WT mice (**Figure 2**). BMDC accumulation increased with BU dosage, whereas accumulation was absent in mice conditioned with vehicle (**Figure 2A**). Similarly, BMDCs accumulated within the lumbar spinal cord of BU-conditioned WT mice in a time-dependent manner for at least 1 year post-BMT (**Figure 2B**). Mice over-expressing mutant human Cu/Zn superoxide dismutase-1 (mSOD) are the most commonly used mouse model of ALS, recapitulating many features of the human disease including neuroinflammation and microgliosis (17). BU conditioning led to a much greater accumulation of BMDCs within the lumbar spinal cord of mSOD mice compared to controls, particularly when mice were conditioned with 125 mg/kg BU (**Figure 3; Table 1**). As BU-conditioned mSOD and WT mice had comparable levels of BM chimerism, this suggests that ALS disease-related signals enhance BMDC accumulation within the CNS.

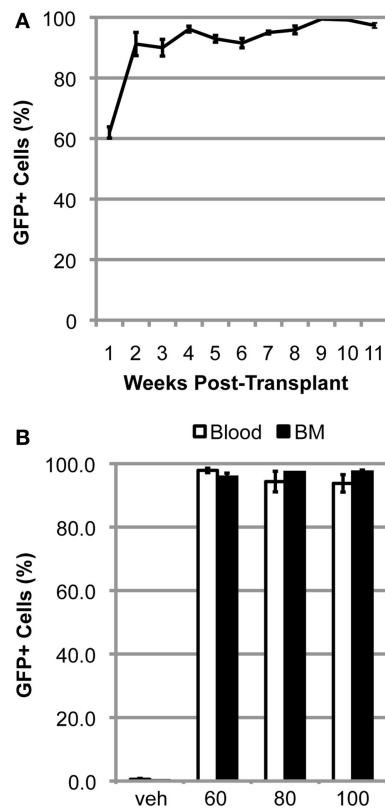


FIGURE 1 | High levels of chimerism are rapidly established and sustained in BU-conditioned mice. (A) Weekly flow cytometric quantification of GFP⁺ cells within the blood in mutant SOD1 mice conditioned with 125 mg/kg of BU and transplanted with GFP⁺ donor bone marrow (BM) cells. **(B)** Flow cytometric quantification of GFP⁺ cells within the blood and BM 1-year post-bone marrow transplantation in wild-type mice conditioned with the indicated dose of BU. Data = mean \pm SEM for $n = 3$ mice.

The morphology of BMDCs can provide insight into both the localization and activation state of the cells. Morphologies were determined according to the criteria outlined by Vallières and Sawchenko, whereby cells were classified as being round, rod, elongated, ameboid, or stellate (28). Generally, cells with stellate and ameboid morphologies are parenchymal in localization while round, rod, and elongated cells are usually located in perivascular regions (28). Very few parenchymal cells were detectable in BU-conditioned WT mice, even when 125 mg/kg was used, suggesting that few cells were able to actually infiltrate the CNS under these conditions (Table 1; Figure 3). Conversely, BU conditioning of mSOD mice led to significantly more BMDC accumulation within the lumbar spinal cord. This was particularly evident when 125 mg/kg BU was used, leading to >50% of the BMDCs accumulating within the lumbar spinal cord exhibiting morphologies that suggest parenchymal localization, with stellate cells being most prevalent (>40%; Table 1; Figure 3). Virtually all of the GFP⁺ BMDCs labeled with Iba1 and CD169, with the only exceptions being a small fraction of round/rod shaped cells (data not shown). These findings suggest that disease-related signals

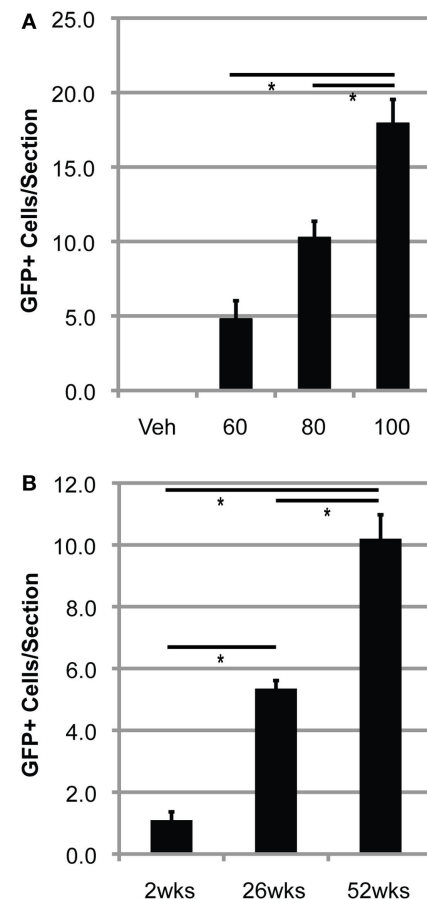


FIGURE 2 | Bone marrow-derived cells accumulate in the lumbar spinal cord of BU-conditioned mice in a time- and dose-dependent manner. (A) Quantification of GFP⁺ donor cells within the lumbar spinal cord 1 year post-bone marrow transplantation (BMT) in wild-type (WT) mice conditioned with the indicated dose of BU (milligrams per kilogram). **(B)** Quantification of GFP⁺ donor cells within the lumbar spinal cord in WT mice conditioned with 80 mg/kg of BU and collected at the indicated times post-BMT. Data = mean \pm SEM for $n = 3$ mice/group; data were analyzed by ANOVA with $p < 0.05$.

are required, along with BU conditioning, in order for BMDCs to gain access and accumulate within the CNS parenchyma rather than in perivascular locations.

A High Level of Chimerism Alone Is Not Sufficient to Produce BMDC Accumulation within the Lumbar Spinal Cord of mSOD Mice

Doses of 60–125 mg/kg BU all led to similar levels of blood and BM chimerism, whereas the extent of BMDC accumulation within the lumbar spinal cord was different, suggesting that a high degree of chimerism alone is not sufficient to result in donor BMDC accumulation within the CNS, and that conditioning of the BBB/CNS is also necessary. To further explore this

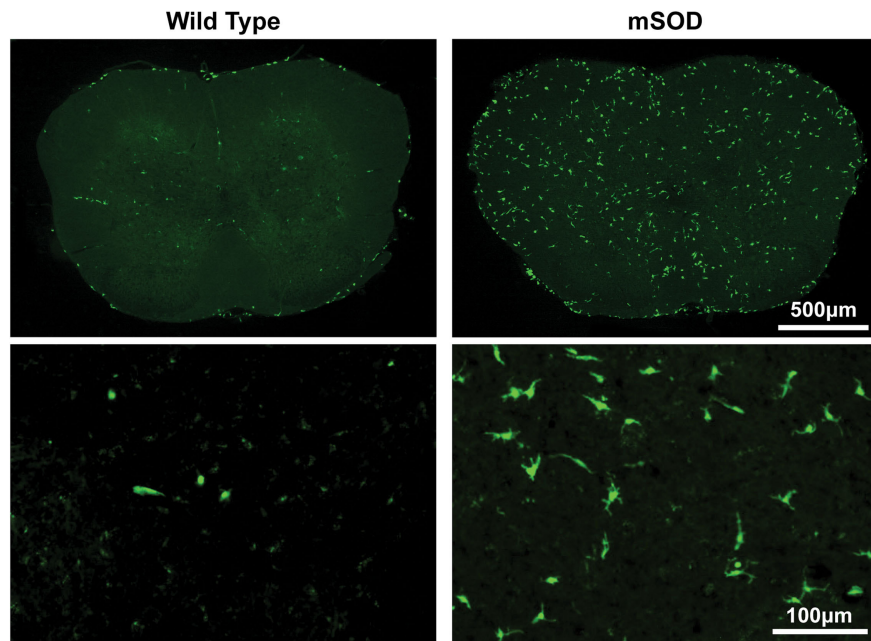


FIGURE 3 | Bone marrow-derived cells accumulate to a much greater extent in the lumbar spinal cord of BU-conditioned mutant SOD1 (mSOD) mice compared to wild-type (WT) controls. Immunohistochemical analysis of lumbar spinal cord sections from late stage mSOD mice and age-matched WT controls that were conditioned with 125 mg/kg BU and transplanted with GFP⁺ donor bone marrow cells at 6–8 weeks of age. GFP⁺ BMDCs are shown in green. Results are representative images from $n \geq 3$ mice.

TABLE 1 | Quantification of GFP⁺ donor cells within the lumbar region of the spinal cord in late stage mutant SOD1 (mSOD) mice and age-matched wild-type (WT) controls transplanted with GFP⁺ donor bone marrow cells at 6–8 weeks of age.

Treatment group	GFP ⁺ cells/lumbar spinal section					
	Total	Round	Rod	Elongated	Ameboid	Stellate
80 mg/kg BU WT ($n = 3$)	4.5 ± 1.0	1.4 ± 0.3 (31.1%)	1.2 ± 0.3 (26.7%)	1.3 ± 0.3 (28.1%)	0.4 ± 0.0 (8.9%)	0.2 ± 0.1 (4.4%)
80 mg/kg BU mSOD ($n = 3$)	55.1 ± 10.7	15.0 ± 4.0 (27.2%)	7.3 ± 0.7 (13.2%)	19.9 ± 3.4 (36.2%)	5.4 ± 1.9 (9.8%)	7.5 ± 1.1 (13.7%)
125 mg/kg BU WT ($n = 5$)	12.5 ± 1.5	1.1 ± 0.3 (8.6%)	5.2 ± 0.7 (41.3%)	5.9 ± 1.0 (47.0%)	0.0 ± 0.0 (0.0%)	0.4 ± 0.1 (2.9%)
125 mg/kg BU mSOD ($n = 4$)	117.9 ± 9.1	3.4 ± 0.4 (2.9%)	25.3 ± 2.3 (21.5%)	25.6 ± 2.5 (21.7%)	13.1 ± 0.4 (11.1%)	50.5 ± 3.8 (42.8%)
6,000 mg/kg TREO WT ($n = 3$)	4.7 ± 0.4	1.6 ± 0.3 (34.0%)	1.7 ± 0.3 (36.9%)	1.4 ± 0.5 (29.8%)	0.0 ± 0.0 (0.0%)	0.0 ± 0.0 (0.0%)
6,000 mg/kg TREO mSOD ($n = 4$)	17.0 ± 1.6	0.5 ± 0.2 (2.6%)	9.7 ± 0.7 (57.1%)	6.5 ± 0.9 (38.2%)	0.2 ± 0.1 (0.9%)	0.2 ± 0.1 (0.9%)

possibility, we established BM chimerism in mice by conditioning with TREO, a hydrophilic analog of BU that does not readily cross the BBB and enter the CNS. Conditioning with 6,000 mg/kg TREO generated a high level of chimerism comparable to that achieved with BU in both the blood and BM, whereas 4,500 mg/kg TREO resulted in a high level of chimerism in the blood but significantly lower levels of chimerism in the BM (Figure 4). Interestingly, TREO conditioning resulted in low levels of BMDC accumulation within the lumbar spinal cord of mSOD mice that were comparable to the levels of BMDC accumulation seen in the lumbar spinal cord of WT mice conditioned with BU (Table 1). Moreover, few stellate and ameboid BMDCs were detectable in the CNS of TREO-conditioned mice, suggesting that the majority of GFP⁺ cells were not localized within the CNS parenchyma.

Mobilization of BM Cells into the Circulation Does Not Increase GFP⁺ BMDC Accumulation within the Lumbar Spinal Cord

Work by Ajami et al. using parabiotic mice suggests that the BM cell type(s) capable of entering the CNS following conditioning and BMT are primitive cells typically restricted to the BM (14). Under physiological conditions, primitive BM cells/progenitors are retained within the BM by stromal cell-derived factor-1 (SDF1), a chemokine with chemoattractant properties for hematopoietic cells expressing the complementary receptor CXCR4 (29). BMDCs were transiently mobilized from the BM into the circulation using either GCSF, a cytokine frequently used clinically to indirectly disrupt SDF1–CXCR4 binding, or

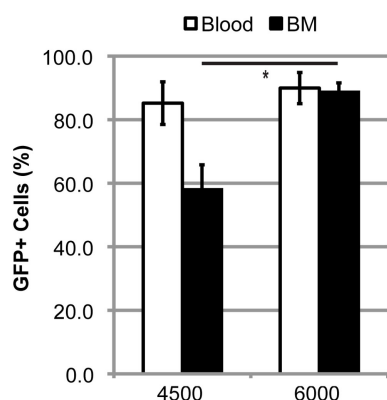


FIGURE 4 | High levels of blood and bone marrow (BM) chimerism are achieved in TREO-conditioned mice. Flow cytometric quantification of GFP⁺ cells within the blood and BM in late stage mutant SOD1 mice that were conditioned with 4,500 or 6,000 mg/kg TREO and transplanted with GFP⁺ donor BM cells at 6–8 weeks of age. Data = mean \pm SEM for $n \geq 3$ mice/group; data were analyzed by a Student's *t*-test with $p < 0.05$.

plerixafor (AMD3100), a small molecule that directly inhibits the SDF1–CXCR4 interaction (**Figure 5A**) (29).

To determine if mobilized BMDCs could accumulate in the CNS and increase GFP⁺ cell number, high levels of stable BM chimerism (>85% GFP⁺ cells) were established for 5 weeks in mice conditioned with 80 mg/kg BU. However, mobilization with either GCSF or AMD3100 did not increase GFP⁺ cell number within the lumbar spinal cord in late-stage mSOD mice and age-matched WT controls (**Figures 5B,C**). We also co-administered GCSF and AMD3100, as gene expression analyses suggest that this combination mobilizes different cell types compared to single drug treatment (30). However, use of both drugs did not result in increased GFP⁺ number within the lumbar spinal cord in late-stage mSOD mice and age-matched WT controls (**Figures 5B,C**). As evidence suggests that BMDC accumulation within the CNS is dependent upon conditioning of the BBB/CNS, we also performed experiments where cells were mobilized 3 days post-BMT in mSOD mice (**Figure 5D**), a timepoint where we hypothesized that the effects of BBB/CNS conditioning would still be present. Even when BM cells were pharmacologically mobilized at this early timepoint, there was no increase in the number of BMDCs accumulating in the lumbar spinal cord of treated mice (**Figure 5D**).

BMDCs Accumulate in the CNS More Rapidly in Mice Conditioned with Higher Doses of BU

To better understand the kinetics of BMDC accumulation within the CNS, we conditioned 15-week-old recipient mSOD mice with either low dose (80 mg/kg) or high dose (125 mg/kg) BU prior to transplantation with GFP⁺ BM cells. Transplanted mice were collected weekly, and the number of GFP⁺ BMDC cells accumulating within the lumbar spinal cord was quantified. During the first 3 weeks post-BMT, very few cells were detectable in the lumbar

region of the spinal cord, regardless of the BU dose used for conditioning (**Figure 6A**). Moreover, the GFP⁺ cells that were detected were primarily rod shaped, with no stellate cells identified, suggesting that these GFP⁺ BMDCs had not yet accumulated within the CNS parenchyma (**Figures 6B,C**). Interestingly, the number of GFP⁺ BMDCs accumulating within the lumbar spinal cord of mSOD mice conditioned with 80 mg/kg of BU remained low for 7 weeks post-BMT and only showed a significant increase when the mice were approaching disease end stage 8 weeks post-BMT (**Figure 6A**). Conversely, the number of BMDCs accumulating within the lumbar region of the spinal cord in mice conditioned with 125 mg/kg BU showed significant increases at weeks 6–8 post-transplant (**Figure 6A**). Importantly, a large proportion of these GFP⁺ BMDCs accumulating within the lumbar spinal cord had a stellate morphology (>50% of BMDCs at weeks 7 and 8 post-BMT), suggesting that these cells were localized within the CNS parenchyma (**Figure 6C**).

BMDC Accumulation within the Lumbar Spinal Cord Is Due in Part to Local Proliferation

To determine whether the accumulation of BMDCs in the lumbar spinal cord was due to proliferation of the GFP⁺ BMDC population, we immunolabeled dividing cells with Ki67, a nuclear protein expressed during mitosis (31). In spinal cords from mice treated with 80 mg/kg BU, Ki67⁺ cells were all GFP[−], except for rare GFP⁺Ki67⁺ cells, indicating that proliferation of the BMDC population was very limited at all timepoints between weeks 3 and 7 post-transplant (**Figure 6D**). In spinal cords from the 125 mg/kg BU group, there were small numbers of GFP⁺Ki67⁺ cells at all timepoints from 4 to 7 weeks post-transplant (**Figure 6D**). The number of GFP⁺Ki67⁺ cells increased significantly in weeks 6 and 7 post-transplant, but cell numbers were small (approximately 5 cells/spinal section; **Figure 6D**). Interestingly, the increase in GFP⁺Ki67⁺ cells paralleled the increase in BMDC accumulation within the lumbar spinal cord (**Figures 6A,D**). Moreover, many of the GFP⁺Ki67⁺ and GFP⁺EdU⁺ BMDCs in the lumbar spinal cord exhibited a stellate morphology (**Figure 6F**), suggesting these cells were located in the CNS parenchyma. By contrast, GFP[−]Ki67 cells were much more evident at all timepoints. These data demonstrate some proliferation of GFP⁺ cells in spinal cord conditioned with 125 mg/kg BU, but that the extent of proliferation of these BMDCs may be limited.

We also evaluated the numbers of Iba1⁺ cells in spinal cord for both the 80 and 125 mg/kg BU groups and found that cell numbers did not increase significantly between weeks 3 and 6 (**Figure 6E**). At week 7, we found a significant increase in Iba1⁺ cells in the 125 mg/kg BU group, but not the 80 mg/kg mice (**Figure 6E**), suggesting that the more extensive conditioning of 125 mg/kg BU may increase the Iba1 cell number. The majority of Iba1⁺ cells in the lumbar spinal cord of mice conditioned with 125 mg/kg BU were GFP[−], measuring 98.5, 91.5, and 72.2% at weeks 3, 5, and 7 post-transplant, respectively. While GFP⁺ cells comprised little of the Iba1⁺ population at weeks 3 and 5 post-transplant (1.5 and 8.5%), by 7 weeks post-transplant GFP⁺ cells were 27.8% of the Iba1⁺ population.

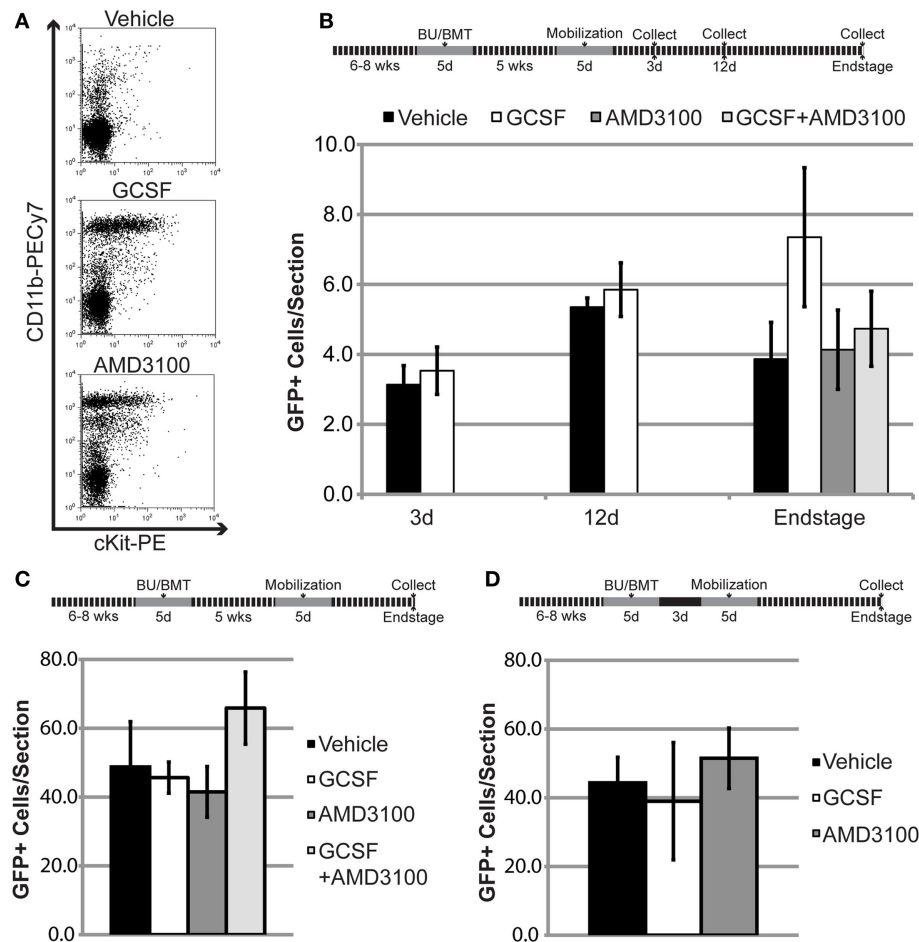


FIGURE 5 | Mobilization of bone marrow-derived cells (BMDCs) into the circulation does not increase BMDC accumulation within the lumbar spinal cord of mutant SOD1 (mSOD) mice and wild-type (WT) controls. (A) Flow cytometric analysis of peripheral blood following mobilization treatments in WT mice. Results are representative images from $n = 3$ mice. **(B)** The 6- to 8-week-old WT mice were conditioned with 80 mg/kg of BU and transplanted with GFP⁺ donor bone marrow (BM) cells. Chimerism was allowed to establish for 5 weeks prior to mobilization treatment. GFP⁺ cells were quantified in lumbar spinal cord sections at the indicated timepoints. Data = mean \pm SEM for $n \geq 3$ mice/treatment. **(C,D)** The 6- to 8-week-old mSOD mice were conditioned with 80 mg/kg of BU and transplanted with GFP⁺ donor BM cells. Chimerism was allowed to establish for 5 weeks **(C)** or 3 days **(D)** prior to mobilization treatment. GFP⁺ cells were quantified in lumbar spinal cord sections at disease end stage. Data = mean \pm SEM for $n \geq 4$ mice/treatment.

DISCUSSION

Conditioning with BU or TREO Produces High Levels of Stable BM Chimerism

Establishment of BM chimerism in animals has typically been achieved using myeloablative doses of irradiation. However, irradiation can lead to secondary complications and alternative approaches to achieving adequate conditioning for BMT have thus been explored (1). We have previously shown that a high degree of BM chimerism can be achieved using myelosuppressive conditioning with the chemotherapeutic molecule BU (3, 11, 12), and these results are supported by several other laboratories (4, 8–10). Consistent with these findings, we have now shown that conditioning with a higher dose of BU (125 mg/kg) followed by BMT leads to rapid establishment of chimerism exceeding 90% in the BM of recipient mice. Moreover, the BM chimerism achieved

using BU conditioning is stable, with high levels maintained for at least 1 year post-BMT. Similarly, conditioning with the BU analog TREO (6,000 mg/kg) produces a high degree of BM chimerism in mice comparable to BU (Figure 4). Our findings are similar to those of Van Pel et al. and Nasa et al., who have shown that concentrations of TREO $\leq 4,500$ mg/kg result in lower, variable levels of chimerism, while 6,000 mg/kg TREO resulted in chimerism of ~80% (22, 23).

These levels of chimerism were achieved when using syngeneic recipients. However, when recipients are not syngeneic, modifications of this conditioning protocol may be needed. For example, in a recent study evaluating the accumulation of BMDCs in the CNS of a murine model of Alzheimer's disease (AD), we transplanted C57BL/6 GFP⁺ BM into mice having a mixed 129/C57BL/6 background. No sustained chimerism was achieved unless anti-asialo ganglio-*N*-tetraosylceramide antibody was used to deplete

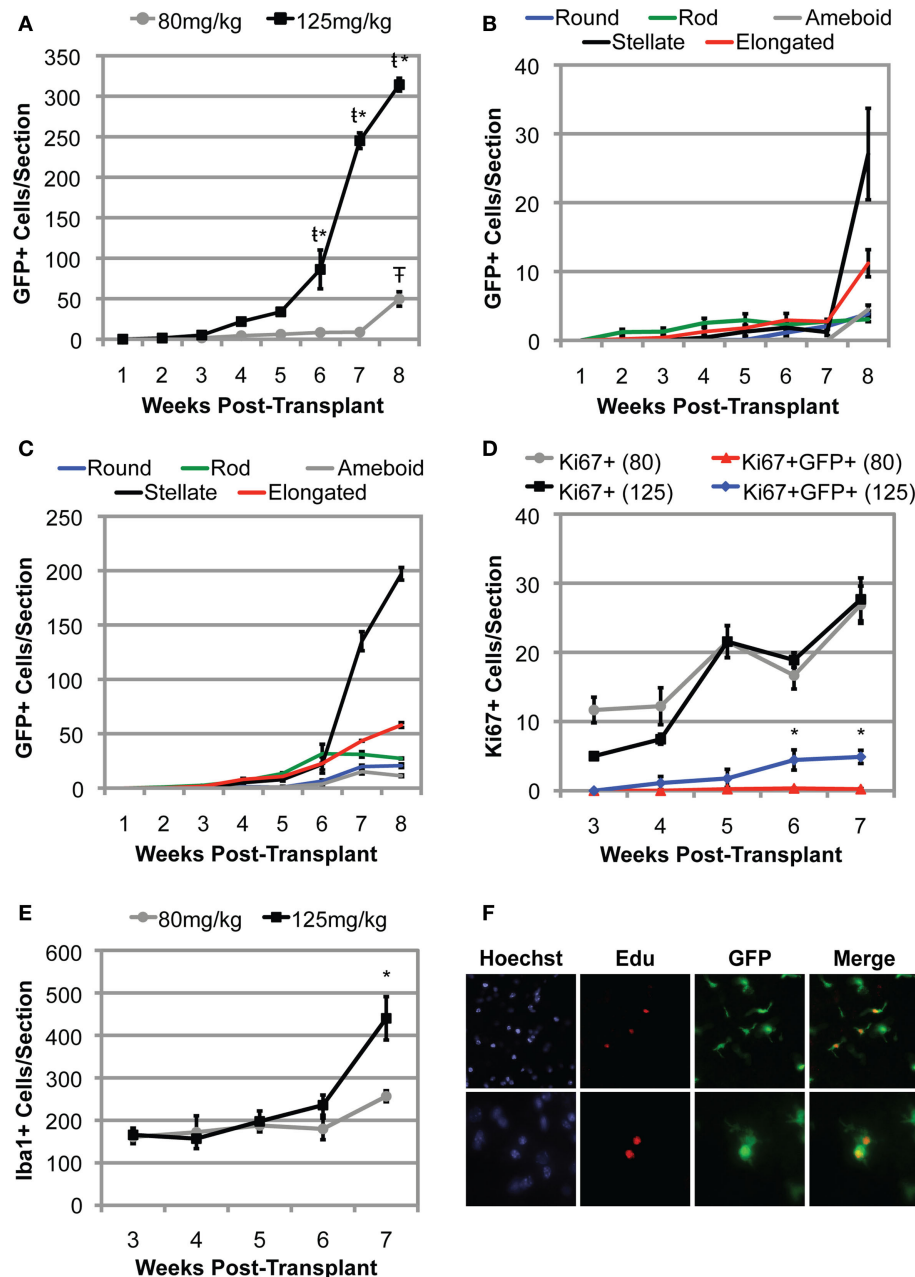


FIGURE 6 | Bone marrow-derived cells accumulate more rapidly and to a greater extent in mutant SOD1 (mSOD) mice conditioned with a high dose of BU compared to a lower dose. (A) Weekly quantification of GFP⁺ cells within the lumbar spinal cord in mSOD mice conditioned with either 80 or 125 mg/kg BU and transplanted at 15 weeks of age. Data = mean \pm SEM for $n = 3$ mice/timepoint. **(B)** Weekly quantification of GFP⁺ cell morphology within the lumbar spinal cord in mSOD mice conditioned with 80 mg/kg BU and transplanted at 15 weeks of age. Data = mean \pm SEM for $n = 3$ mice/timepoint. **(C)** Weekly quantification of GFP⁺ cell morphology within the lumbar spinal cord in mSOD mice conditioned with 125 mg/kg BU and transplanted at 15 weeks of age. Data = mean \pm SEM for $n = 3$ mice/timepoint. **(D)** Weekly quantification of Ki67⁺ cells and GFP⁺Ki67⁺ cells within the lumbar spinal cord in mSOD mice conditioned with either 80 or 125 mg/kg BU and transplanted at 15 weeks of age. Data = mean \pm SEM for $n = 3$ mice/timepoint. **(E)** Weekly quantification of Iba1⁺ cells within the lumbar spinal cord in mSOD mice conditioned with either 80 or 125 mg/kg BU and transplanted at 15 weeks of age. Data = mean \pm SEM for $n = 3$ mice/timepoint. **(F)** Immunohistochemical analysis of lumbar spinal cord sections from late stage mSOD mice that were conditioned with 125 mg/kg BU and transplanted with GFP⁺ donor BM cells at 15 weeks of age. Mice were given IP injections of 50 mg/kg 5-ethynyl-2'-deoxyuridine (EdU) 48 and 24 h prior to collection to label proliferating cells. Nuclei are shown in blue, EdU in red, and GFP⁺ cells in green. Results are representative images from $n = 3$ mice. Data were analyzed by ANOVA, and *post hoc* tests using the Tukey-Kramer method were used. * represents a significant difference between 80 and 125 mg/kg treatment groups with $p < 0.01$; † represents a significant difference from the preceding timepoint with $p < 0.01$; ‡ represents a significant difference from the preceding timepoint with $p = 0.02$.

natural killer cell activity, likely due to “hybrid resistance” caused by autoreactive natural killer cells from the mixed background recipient being directed to the transplanted BM (12). With BMT into syngeneic mice, no graft rejection was observed.

CNS Conditioning Is Required for BMDC Accumulation within the CNS

High levels of BM chimerism (>85%) were achieved using BU doses of 60–125 mg/kg. However, despite having comparable levels of chimerism, BMDCs accumulated within the lumbar spinal cord in a dose-dependent manner. These results indicate that establishment of high levels of chimerism alone does not result in the accumulation of BMDCs within the CNS. In addition, when high levels of BM chimerism were achieved using TREO, low numbers of BMDCs were detectable within the lumbar spinal cord. Based upon its hydrophilicity, TREO would not be predicted to readily cross the BBB and gain access to the CNS. In support of this claim, little penetrance of TREO and its active metabolites were detectable within the CNS of rats administered 500 mg/kg TREO, and limited amounts of TREO crossed an *in vitro* BBB model (20, 21). Virtually, all of the BMDCs detectable in TREO-conditioned mice had morphologies suggesting blood vessel-associated localizations (Table 1). The very small proportion of the BMDCs with stellate or amoeboid morphologies detected within the lumbar spinal cord of TREO-conditioned mice (<2%) is likely due to limited TREO penetration of the BBB and CNS conditioning. These results suggest that some degree of BBB disruption and/or CNS conditioning also likely needs to be present, either by irradiation or chemotherapy. BU has been shown to cross the BBB (~20% of a dose administered in humans) (32), and evidence suggests that the BBB is not obviously disrupted by BU conditioning (10). In crossing the BBB, BU may be creating niche space within the microglial compartment allowing for BMDCs to infiltrate the CNS and accumulate (8). Alternatively, as high doses of BU have been shown to have neurotoxic effects in patients and animals (33), it is also possible that BU is causing neuronal damage, which in turn increases BMDC accumulation through increased recruitment of BMDCs from the circulation and/or stimulating local BMDC proliferation within the CNS. Unlike the findings of Butovsky et al. (18), the present data indicate that in the absence of a CNS conditioning stimulus, there is limited entry and accumulation of BMDCs in the spinal cord, even in diseases such as murine ALS.

BMDC Accumulation within the CNS Is Dependent on Disease-Related Mechanisms

Small numbers of BMDCs were observed in WT mice conditioned with BU. Moreover, the morphology of BMDCs accumulating within the lumbar spinal cord of WT mice suggest that few, if any, of the cells were located within the CNS parenchyma. These GFP⁺ BMDCs were likely perivascular, even when 125 mg/kg BU was used. Recent studies by Wilkinson et al. found that 125 mg/kg BU caused considerable accumulation of stellate BMDCs within the CNS of WT mice, an effect that was larger than that caused by myeloablative irradiation (4). Using the same dose of

BU conditioning, we found substantially lower numbers of GFP⁺ BMDCs in the lumbar spinal cord and fewer GFP⁺ cells with a stellate morphology than Wilkinson and colleagues (Table 1). The differences we see in BMDC accumulation compared to Wilkinson et al. may be due to different CNS tissue being analyzed (brain vs spinal cord) or a slightly different transplantation procedures where we transplanted 10-fold less donor BMDCs (4).

As in many previous studies using both irradiative and non-irradiative conditioning, we find much larger numbers of BMDCs in the lumbar spinal cord of mSOD mice compared to WT mice, an effect that is also seen in other models of neurodegenerative disease (Table 1; Figure 3) (3, 11, 12). Stellate and amoeboid cells generally constitute parenchymal cells within the CNS (28) and cells with these morphologies were frequently seen in mSOD mice, while very few were observed in WT mice, suggesting that disease-related mechanisms were involved in BMDC accumulation within the CNS. Notably, the majority of BMDCs in the lumbar spinal cord of late stage mSOD mice conditioned with 125 mg/kg BU had a stellate morphology (Table 1), while our previous work using myeloablative irradiation showed the majority of cells to have an amoeboid morphology (3). These differences in BMDC morphology between irradiation and BU may be related to the cytokine profile triggered by the conditioning treatment (3).

From previous work, it is known that the BMDCs that accumulate in the CNS are mostly monocytic lineage cells that express CX₃CR1, although small numbers of T lymphocytes may be present (2, 27, 34, 35). It is likely that endogenous microglia and monocyte-derived CNS macrophages constitute distinct populations with different functions (36, 37), but that the properties of these cell populations will differ in neurodegenerative disorders such as ALS or diseases with prominent inflammation, such as experimental autoimmune encephalitis (EAE). In EAE, monocyte lineage cells efficiently enter the CNS and give rise to mature macrophages that seem indistinguishable morphologically from resident microglia (38). However, as recovery from the acute phase of EAE occurs, infiltrating monocyte lineage cells which had accumulated in the CNS undergo apoptosis (38). In contrast, the extent of BMDC accumulation in models of chronic neurodegenerative diseases such as ALS increases, likely due to expansion of accumulated BMDCs and possibly further BMDC entry. The factors responsible for BMDC entry include CCR2, as limited BMDC accumulation of BMDC occurs in CCR2^{-/-} mice (38). Based upon work in EAE, it has been claimed that monocyte-derived macrophages are phagocytic and inflammatory, whereas resident microglia have less inflammatory properties based on gene expression data (37). For instance, monocytes demonstrate an upregulation of CXCR2, CCR1, toll-like receptor 6, and other genes associated with inflammation, whereas microglia upregulate TNF, Stat1, and other genes associated with metabolism (37). It is likely that the method of chimerism used to achieve BMT in such studies will be relevant for the profile of gene expression observed. In mice transplanted using BU, elevations are seen in levels of serum and CNS G-CSF and IL-6 shortly following BMT, compared to irradiation (4). Monocyte lineage cells ultimately derive from the monocyte/macrophage and dendritic cell precursor (MDP) (13). There is also evidence for a clonogenic

monocyte/macrophage restricted progenitor cell derived from the MDP termed the common monocyte progenitor (39) which could, in principle, be a cell type that accumulates in the CNS following BU administration and BMT.

Mobilization of BM Cells into the Circulation Does Not Increase BMDC Accumulation in the CNS

During BMT using whole or fractionated BM, BM cells including progenitors are injected into the circulation of the recipient animal and may accumulate within the CNS. In an attempt to increase BMDC accumulation within the CNS, we mobilized cells from the BM using GCSF, AMD3100, or a combination of both treatments. Despite mobilizing BM cells (**Figure 5A**), we were unable to increase the amount of BMDC accumulation in the lumbar spinal cord. It is possible that the BM cells mobilized by GCSF and AMD3100 were not capable of migrating into the CNS. Alternatively, BM cells in the blood circulation may not have been mobilized for sufficient time, or in sufficient numbers to accumulate within the CNS. While there is evidence showing that GCSF can increase the number of BMDCs accumulating in the CNS of a mouse model of AD, these treatments were typically combined with other factors such as SDF-1, which would be expected to influence BMDC migration (26, 40). Furthermore, these experiments were performed in irradiated mice which may lead to different CNS/BBB conditioning that allows for the accumulation of mobilized cells within the CNS. These observations have implications for the treatment of ALS. Previous studies have used GCSF treatment in ALS patients to produce BM mobilization for collection and re-injected into patients to in an attempt to generate BMDC accumulation in the CNS (41, 42). However, given the present results, it remains doubtful as to whether GCSF treatment will alter BMDC accumulation in the CNS.

BMDC Accumulation and Proliferation

The weekly analysis of BMDC accumulation within the lumbar spinal cord indicates that following myeloablation and chimerism, only limited cell accumulation occurs for several weeks. A substantial increase in cell accumulation occurs only after the first 5 weeks post-transplant in mSOD mice conditioned with 125 mg/kg BU and after 7 weeks in mice conditioned with 80 mg/kg BU. This increase in cell number corresponds to a time when mice are developing neurological deficit, raising the possibility that the increased cell number occurs in relation to disease-related signals. It is well known that microglial proliferation occurs in mSOD mice (43). It is possible that the GFP⁺ BMDCs that seeded the CNS proliferate at a similar rate of expansion as endogenous microglia and that this expansion is responsible for some of the large numbers of GFP⁺ BMDCs seen, especially in mSOD mice at later times following transplantation (23). However, the present results show only small numbers of Ki67⁺GFP⁺ cells suggesting that in addition to cell proliferation, some entry of BMDCs likely occurs and that this entry is likely dependent on the conditioning stimulus.

It remains unclear to what extent BMDC accumulation within the CNS of BU-conditioned mice is due to proliferation compared to continued infiltration. In experiments using the proliferation

markers EdU or Ki67, we found cell labeling of stellate GFP⁺ cells within the lumbar spinal cord. However, the number of cells labeled with these markers was small, consistent with the findings of Wilkinson et al. (4). As we only administered EdU for 48 h prior to collecting tissue, it is unlikely that the cells divided in the BM and subsequently migrated into the lumbar spinal cord parenchyma. Moreover, several of the EdU-labeled cells we observed appeared to be actively dividing (**Figure 6F**), and Ki67 only labels cells that are proliferating. The limited numbers of cells labeled by EdU is likely a consequence of the short-time course that EdU can be administered to minimize the possibility of labeling proliferating within the BM that could subsequently infiltrate the lumbar spinal cord. Moreover, Ki67 only labels cells that are actively proliferating at the time of fixation and tissue collection. Thus, it is likely these techniques underestimate the extent that proliferation accounts for BMDC accumulation within the CNS.

CONCLUSION

The use of BMDCs in the therapy of CNS disease is in its infancy. To be clinically useful, BMDCs will need to be administered with conditioning agents that are mild enough to be tolerated, yet permit long-term BM chimerism. BU appears to be useful for this aim. Furthermore, this work and others have shown that high-level blood and BM chimerism are insufficient to ensure that large numbers of BMDCs will accumulate in the CNS. The results also show that substantially more BMDC accumulation can occur in a disease model than in WT animals, indicating that disease-related factors will influence BMDC entry, likely in a time-dependent manner. We do not find that mobilization of BM by GCSF or AMD3100 influences the extent of BMDC accumulation in CNS but suggests that BMDCs that accumulate in the CNS increase in number partially through proliferation.

AUTHOR CONTRIBUTIONS

KP, JM, C-AL, FR, and CK conceptualized the experiments. All experiments were performed by KP with the assistance of JM and KT. The manuscript was written by KP and revised by C-AL, FR, and CK.

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