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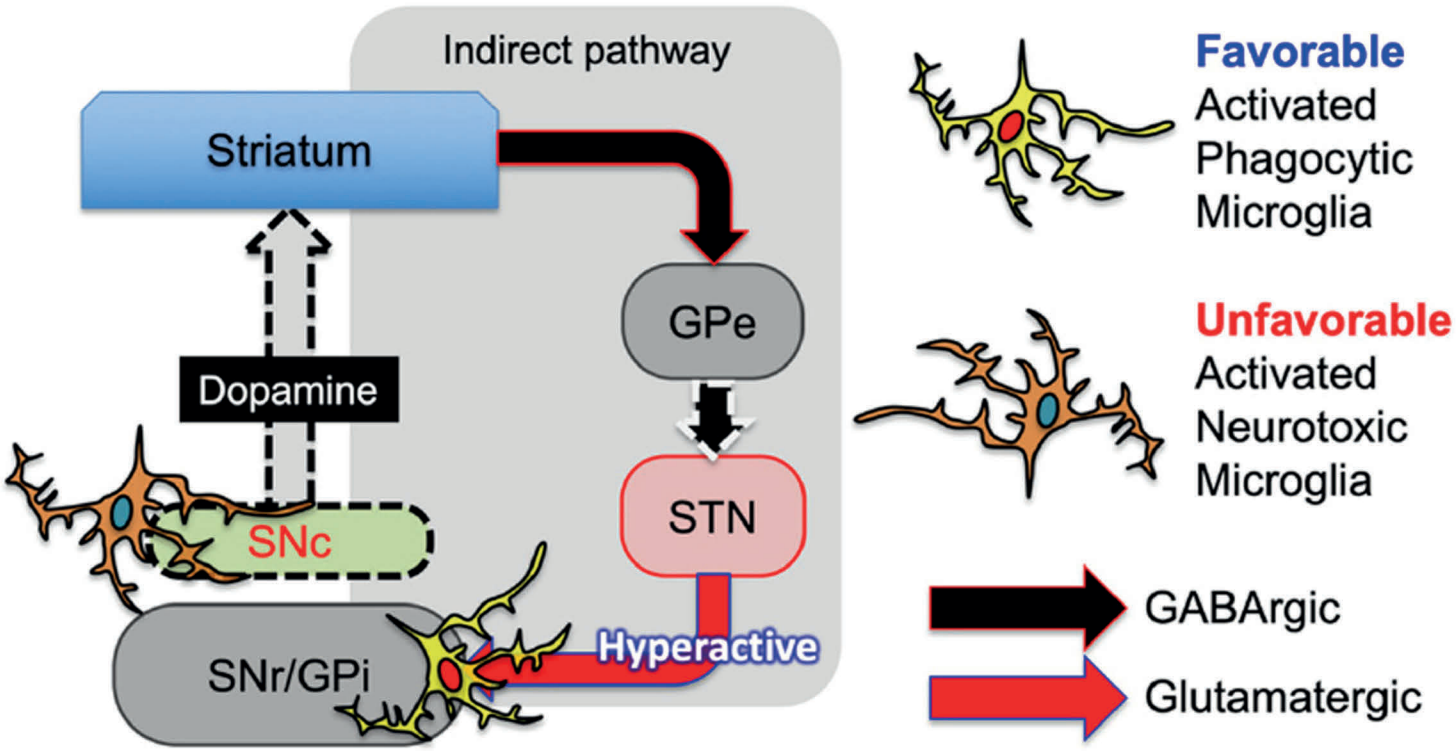

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CONTENTS

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Neuroimmunology and Neuroinflammation

Review

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Favorable and unfavorable roles of microglia and macrophages in the pathologic central nervous system

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Abstract

Resident microglia in the central nervous system (CNS) are activated rapidly in response to even minor pathologic changes in the CNS, releasing various cytokines, growth factors, reactive oxygen species and other bioactive substances, in addition to eliminating synapses and degenerating cells through phagocytosis. Monocytes in circulation invade the inflamed brain tissues and develop into macrophages that also produce several bioactive substances and engage in phagocytosis. This article introduces methods for distinguishing microglia and macrophages. The pathophysiological roles of resident microglia and macrophages are discussed in animal models with neuroinflammation in the brain either with or without disruption of the blood-brain barrier. Both cell types have ameliorating and aggravating effects on the pathologic CNS, and their different roles are addressed in this article. Furthermore, this article compares the effects of some pharmacological interventions to induce phenotypic cellular changes for improved outcomes of the pathologic CNS.

Keywords: Parkinson's disease, stroke, traumatic brain injury, axotomy, spinal cord, glucocorticoid, noradrenaline, bromovalerylurea

INTRODUCTION

Microglia and blood-borne macrophages play major roles in the pathophysiological processes in various kinds of pathologies of the central nervous system (CNS) by releasing numerous bioactive substances,

including cytokines, growth factors, and reactive oxygen/nitrogen species, and phagocytosing degenerating cells and materials[1-4]. In this article, we use "microglia" to denote resident microglia in the CNS, and "macrophages" to denote cells derived from circulating monocytes that have invaded (typically inflamed) CNS lesions with a disrupted blood-brain barrier (BBB). The two types of mesoderm-derived cells share many kinds of characteristics and surface antigens[5,6]. In particular, when microglia become activated in response to severe pathologic events, such as stroke or traumatic brain injury (TBI), which accompany BBB breakdown, both cell types resemble each other in terms of morphology, functions, and cell marker expression. The term "amoeboid microglia"[7] has long been used to denote extremely activated microglia displaying almost the same morphology as brain macrophages in severe lesions. Therefore, it is generally difficult to distinguish one from the other. This assumption originated from the historical observation by Rio-Hortega[8], who reported that ramified microglia in the normal mature brain can, in severely damaged brains, turn into phagocytes exhibiting spherical shapes of the same morphology as that of blood-borne macrophages[5]. Therefore, numerous studies have described CD11b-expresssing (in the case of the rat brain, a monoclonal antibody OX-42-immunoreactive) spherical cells in severely injured brains in the acute phase as activated microglia, although most of them should be recognized as invading neutrophils[9,10].

More recently, it is well-known that both cell types play major roles in pathophysiological processes[2,11,12]. The neuroinflammatory processes influence outcomes of CNS diseases or injuries in both favorable and unfavorable ways. In this review, "favorable" is used to describe microglia and macrophages that are neuroprotective cells bringing about the better outcome in the pathologic CNS than "unfavorable" ones that exert deleterious effects on the survival of neurons and other parenchymal cells. Therefore, various kinds of interventions, including pharmacological treatment and rehabilitation, have been studied in laboratory and clinical settings to determine if they enhance favorable responses while also suppressing the deleterious effects of microglia and macrophages[1,4,13,14]. This aim may be interpreted as attempting to induce M2-polarized or alternatively activated phenotypes of these cells[15-17]. However, phenotypes of activated microglia and macrophages cannot be classified clearly into M1-polarized or M2-polarized cells[18,19].

Diseases and injuries of the CNS can be categorized by the absence of BBB disruption[20,21]. In the absence of BBB disruption, the infiltration of circulating leukocytes is limited, and microglia play a central role as immune cells. When the BBB is disrupted, infiltrating leukocytes play much more significant roles[9,22]. In particular, invading monocytes increase hugely in number because of their strong proliferative nature and end up occupying almost the entire area of the lesion in either stroke or traumatic injury[11,12]. Marked accumulation of blood-borne macrophages is seen in malignant brain tumor masses, which are termed tumor-associated macrophages[23,24]. Tumor-associated macrophages contribute to tumor growth and angiogenesis by secreting many kinds of growth factors, including vascular endothelial growth factor. Tumor-associated macrophages are not discussed in this article.

Thus, microglia and macrophages are the critical cells in CNS diseases and injuries and have a profound impact on patient outcomes. Therefore, many studies have investigated interventions to both suppress the unfavorable effects of microglia and macrophages and induce their favorable functions. This article also deals with several interventions targeting microglia and macrophages. There are three types of resident macrophages that are distinct from microglia and the invaded blood-borne macrophages. Those are the meningeal, perivascular and choroid plexus macrophages. Although they play significant roles in health and pathology, they are not discussed in this review.

DISTINGUISHING BETWEEN MICROGLIA AND MACROPHAGES

Discriminating microglia from macrophages has been considered to be very difficult. This is partly due to the old notion that microglia in the brain are derived from circulating monocytes[7,25]. Moreover, microglia

have long been thought to be able to display almost completely spherical or amoeboid morphology when they become fully activated in the core of severe brain insults such as stroke or trauma[5]. Both cell types share numerous immune cell markers[26]. Therefore, distinguishing between them has been very problematic. However, many methods have been now established for this purpose.

Morphological characteristics

Despite the historical view, even highly activated microglia do not display spherical morphology[9,27]. In a rat stroke model prepared by transient occlusion of middle cerebral artery (tMCAO), activated microglia in the peri-ischemic regions exhibit enlarged somata and shortened processes that are distinct from spikes. The processes can be identified with immunoreactivity to microglia/macrophage markers such as Iba1 or CD11b. Conversely, blood-borne macrophages and neutrophils, the latter of which infiltrate in abundance, do not have processes even though they may have a polygonal shape and short spikes[27]. Thus, microglia can be distinguished from blood-borne cells via morphological observation[10]. This may be the simplest method for specific identification of microglia.

Specific markers to distinguish the two cell types in the pathologic brains

Iba1, CD11b, CD45, and CD68 have long been used to identify resident microglia; however, these markers are more strongly expressed by infiltrating macrophages than by activated microglia[9]. As CD11b is a marker for myeloid cells and CD45 is a marker for all of the leukocytes, they are not suitable for identifying microglia in the pathologic CNS. To examine specific roles of resident microglia in CNS pathology, they must be distinguished from blood-borne cells that are macrophages, lymphocytes, and neutrophils. Thus, considerable effort has been dedicated to finding microglia-specific markers that are not expressed by macrophages and other blood-borne cells[28]. Comprehensive gene analyses using RNAseq and/or microarray analyses have identified genes that are expressed predominantly by microglia rather than by macrophages such as *Cx3cr1, Gpr34, P2ry12*, *P2ry13*, *Siglech*, *Tmem119*, and *Trem2*[29-32]. Of the specific marker candidates, TMEM119 and Siglec-H may be the most promising for immunohistochemical discrimination of microglia from macrophages.

Transmembrane protein 119, commonly known as TMEM119, was identified by Bennet *et al.*[33] Specific expression of TMEM119 by microglia has been demonstrated by immunohistochemical staining, flow cytometry analyses, and *in situ* hybridization[33,34]. TMEM119 was identified originally as a type I transmembrane protein expressed by murine osteoblasts and is responsible for their differentiation[35]. It is not expressed by microglia in immature murine brains, but its expression increases along with their development or ramification[33]. In aged human brains either with or without Alzheimer's disease (AD), microglia expressed TMEM119[34]. Activated microglia with enlarged somata in close proximity to amyloid plaques are less immunoreactive to TMEM119 antibody than are resting (or homeostatic) microglia. Moreover, TMEM119-expressing microglia in AD brains either do not or only weakly express the polarized markers CD80, CD163, or CD206. In a TBI model, ramified or homeostatic microglia express TMEM119 at higher levels than do activated microglia[10]. Therefore, TMEM119 is particularly suitable for identifying homeostatic microglia with a ramified shape in both the normal and the injured mature brain.

Sialic-acid-binding immunoglobulin-like lectin-H (Siglec-H) is another promising marker for immunohistochemical discrimination of microglia from macrophages[36]. Siglec-H is a single-pass transmembrane protein that was identified originally as a member of a CD33-related Siglec family. Siglec-H is barely expressed by circulating monocytes and their derived macrophages. In contrast to TMEM119, Siglec-H is expressed continually by activated microglia and by microglia in immature brains. Siglec-H may mediate signals necessary for phagocytosis by microglia[37].

Bone marrow transplantation

Bone marrow transplantation (BMT) has long been a reliable method for identifying blood-borne cells in the CNS with BBB breakdown[28]. After ~10 Gy irradiation to cause near-total death of the host's bone

marrow, the bone marrow from transgenic animals that ubiquitously express fluorescent proteins such as enhanced green fluorescent protein is transplanted[12]. In the transplanted brain, blood-borne macrophages bear fluorescence but not resident microglia[27]. However, BMT usually leads to partial chimera, which can make it difficult to analyze the results. With reconstruction of the bone marrow, it is a long time before the anemia disappears. Radiation may cause degeneration of neural cells, such as NG2 glia and neurons, which may affect the results[38]. As radiation disrupts the BBB while increasing monocyte infiltration, BMT produces donor-derived microglia or microglia-like ramified cells in the brain parenchyma[28]. Thus, the BMT may provide firm evidence showing the presence of blood-borne cells in the CNS, but it should be noted that BMT itself will significantly change the brain functions due to the toxic effects on the neural cells.

Flow cytometry

Microglial cells belong to a group of myeloid leukocytes and express a macrophage colony-stimulating factor receptor known as CSF-1R. Therefore, microglia express a myeloid cell marker CD11b and a pan-leukocyte marker CD45. Based on this finding, microglia have been analyzed by flow cytometry using antibodies to CD11b and CD45[10,39]. Because of faint expression of CD45 by homeostatic microglia, immunohistochemical detection of this expression tends to be difficult. Conversely, macrophages and neutrophils express CD45 rather strongly[10]. However, weak CD45 expression can be detected easily by flow cytometry and is an advantage in flow cytometry analysis; microglia can be defined as $CD11b^{+}$/$CD45^{lo}$ and macrophages as $CD11b^{+}$/$CD45^{hi}$. Even activated microglia express at lower levels of CD45 than do macrophages. Moreover, activated and homeostatic microglia can be distinguished by flow cytometry based on forward and side scatter value; activated microglia have larger somata (larger forward scatter values) and more intracellular organelles (larger side scatter values) than do homeostatic microglia[10]. Flow cytometry analyses can be used to isolate the cells by cell sorting[10,39]. If the cells are treated appropriately to prevent degradation of proteins, RNA, or DNA, the sorted cells can be used for either Western blotting or PCR.

Flow cytometry analyses of either cultured microglia or circulating monocytes are simple[40,41]. Nevertheless, analyses of microglia and macrophages in brain tissues have been difficult because of difficulties in dissociating the tissues into single cells. However, dissociation kits and apparatus are now available for preparing neural cell suspensions and are designed appropriately for dissociation of rodent and human brains[10,39]. The sorted cells can also be used for culturing and/or functional analyses.

RESPONSE OF MICROGLIA IN BRAIN PATHOLOGY IN THE ABSENCE OF BBB BREAKDOWN

Microglia become activated in response to even minor pathological events that do not involve BBB disruption. This section discusses microglia in Parkinson's disease (PD), peripheral nerve injury, and Carbon monoxide (CO) intoxication. Minute activation of homeostatic microglia accompanying circadian changes can be observed in the normal mature brain; microglia exhibit weakly activated phenotypes around the time of onset of sleep[39].

PD

PD is the second most frequent neurodegenerative disorder after AD. As BBB breakdown is not apparent in PD, infiltration of leukocytes, including monocytes, is not often seen. In PD pathophysiology, dopaminergic (DA) neurons in the substantia nigra pars compacta (SNc) in the mesencephalon primarily undergo degeneration, leading to microglial activation in the vicinity of the degenerating neurons[42-44]. The activated microglia release potentially neurotoxic substances, such as either reactive oxygen/nitrogen species or glutamate[45,46]. The microglia-derived proinflammatory cytokines and chemokines may also contribute to aggravation. Injection of 6-hydorxydopamine (6-OHDA) into the striatum or medial forebrain bundle is used to prepare the PD rat model[47,48]. In the model, DA neurons in the SNc primarily

undergo degeneration, and microglia then become activated in response to damage-associated molecular patterns (DAMPs), such as high mobility group box-1 protein (HMGB1) released from damaged neurons[49]. The activated microglia may exacerbate the degenerative processes of DA neurons. Another rat PD model is prepared via injection of lipopolysaccharide (LPS) either direct into the SNc or in its vicinity[48]. In this model, microglial activation is primarily induced followed by DA neuron degeneration, suggesting that activated microglia could be a key cause of neuronal degeneration.

The hematopoietic cytokines interleukin-3 (IL-3) and granulocyte/macrophage colony-stimulating factor (GM-CSF) have been shown to modulate the phenotype of microglia in the SNc while suppressing proinflammatory nature and increasing secretion of neurotrophic factors, insulin-like growth factor 1 (IGF-1), and hepatocyte growth factor (HGF)[44]. Following this, DA neurons increase the expression of anti-apoptotic factor Bcl-xL, and the symptoms of PD are ameliorated. This suggests that further investigation of microglial phenotypes would lead to a potential intervention for neurological disorders.

Microglia in the SNc have been the sole focus of studies investigating microglia in PD pathology. However, upon immunostaining sections of the mesencephalon of PD model rats with antibodies to microglia markers, activation of microglia in the substantia nigra pars reticulata (SNr) was more apparent than that of those in the SNc[47]. The activated microglia in the SNr bore large $CD68^+$ phagosomes in their cytoplasm, in which synaptic proteins were included. In the PD pathology, glutamatergic neurons in the subthalamic nuclei (STN) become hyperactivated and release excess amounts of glutamate in the basal ganglia outputs that are the SNr and the internal segment of the globus pallidus (GPi). The change causes bradykinesia, rigidity, and other PD symptoms. Activated microglia with large phagosomes are present not only in the SNr but also in the GPi. They internalize the glutamatergic synapses from the hyperactive STN. As the neurological deficits do not manifest until most DA neurons are lost, it is likely that there are some significant compensatory mechanisms that prevent the symptoms from appearing[50]. Microglia should contribute to this compensation by eliminating hyperactive glutamatergic synapses. Administration of a single high dose of a synthetic glucocorticoid dexamethasone (Dex) to the PD model rats aggravated their motor deficits. Dex suppresses CD68 expression in the SNr and GPi, suggesting suppression of microglial phagocytosis.

Conversely, chronic administration of glucocorticoid[51,52] and other anti-inflammatory agents, such as bromovalerylurea (BU)[43], has been shown in laboratory settings to ameliorate the outcome of the motor deficits, likely because of suppression of proinflammatory activation of microglia in the SNc. These findings reveal that microglia play both ameliorative and detrimental roles. There appear to be two forms of microglia activation: one is characterized by the production of proinflammatory mediators found in the SNc[43,44], and the other is characterized by enhanced phagocytic ability in the SNr and GPi[47]. The dual role of microglia in the PD pathophysiology is summarized in a schematic diagram [Figure 1].

Despite significant evidence showing the involvement of microglia in the DA neuron loss in animal PD models, it is not clear whether microglia actually affect the pathology of human PD cases. A few clinical trials have shown the positive effects of antiinflammatory drugs[53], and activated microglia are found in PD patients' brains by *in vivo* imaging with positron emission tomography[54]. However, there is still no firm evidence demonstrating that microglia actually induce DA neuron death in human PD cases. Most anti-inflammatory interventions are not ameliorative[55,56]. This contrasts with animal model cases, in which anti-inflammatory interventions markedly suppress DA neuron death. The discrepancy between animal PD models and human cases may be partially attributable to the speed of the pathological processes. The animal model is acutely prepared, whereas human PD is a chronic disease. Furthermore, when PD is diagnosed based on motor symptoms, more than 60 % of DA neurons in the SNc are degenerated. In animal models, anti-inflammatory drugs are often given simultaneously with or even before administration

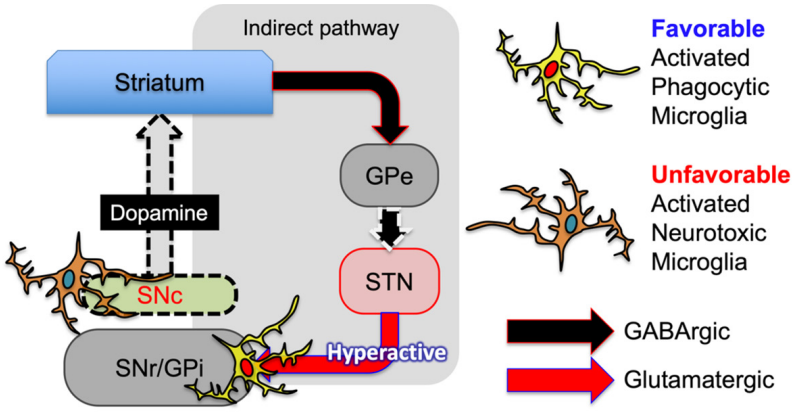


Figure 1. Both favorable and unfavorable activated microglia participate in the pathophysiology of PD. Unfavorable microglia present in the SNc release neurotoxic proinflammatory mediators while accelerating the degeneration of DA neurons. Favorable microglia present in the SNr and GPi engage in eliminating hyperactive glutamatergic synapses from the STN in the indirect pathway of the basal ganglia. This figure is based primarily on the study by Aono *et al*.[47]. PD: Parkinson's disease; SNc: substantia nigra pars compacta; DA: dopaminergic; SNr: substantia nigra pars reticulata; GPi: globus pallidus; STN: subthalamic nuclei; GPe: globus pallidus pars externa

of neurotoxic substances causing DA neuron loss. Even if anti-inflammatory treatments could delay or prevent progressive neuronal loss in PD pathology, it is a prerequisite for the treatments that diagnosis is made at much earlier time points when most DA neurons in the SNc are still viable.

Peripheral nerve injury

Peripheral nerve injuries induce activation of microglia in close proximity to damaged neurons. In particular, facial nerve transection has been used frequently to observe the response of microglia[57]. Microglia become activated and proliferate while attaching intimately to the axotomized neurons. Direct contact with neurons may be one of the direct causes of the microglial activation[58]. The activated microglia detach afferent axonal endings through "synaptic stripping"[57,59]. In the axotomy model, the activated microglia may be neuroprotective through releasing a plethora of neuroprotective factors[60]. Axotomy and spinal cord ischemia both caused similar changes of microglia around the alpha motoneurons in the anterior horn[61]. Synapses surrounding the neurons disappeared when activated microglia attached intimately to damaged neurons.

Constriction injury of the sciatic nerve is another model that is used often to study the responses of microglia in the spinal cord. This model is well known to cause chronic neuropathic pain[62]. Microglia exhibit activated morphology in the posterior horn, and they phagocytose myelin elements[63]. Constriction injury-induced hyperalgesia and the activation of microglia in the posterior horn are sustained chronically. Microglia in the anterior horn also become activated while surrounding damaged motoneurons and remove afferent synapses, as do microglia do in the facial nerve axotomy model. Different from the chronic sensory impairment, Constriction injury-induced motor deficits become ameliorated quite rapidly, indicating that the activated microglia in the anterior horn may be neuroprotective. Thus, microglia could become either protective/favorable or destructive/unfavorable cells.

CO intoxication

CO intoxication causes serious adverse effects in brain functions that are known as delayed encephalopathy. Compared with hypoxia-induced disorder, CO intoxication causes more severe memory impairment and more aggravated degeneration of neuronal cells in the hippocampus[64]. Moreover, CO intoxication causes damage of oligodendrocytes, myelin and NG2 glia. Notably, CO intoxication induces profound loss of microglia. Expression of neurotrophic factors, such as IGF-1, HGF, platelet-derived growth factor, and

basic fibroblast growth factor (bFGF) is reduced considerably, whereas expression of pro-apoptotic factors, such as Bid, Bad, and Bax, is increased, leading to marked loss of the hippocampal neurons. These findings indicate that microglia contribute significantly to supporting neuronal survival.

RESPONSE OF MICROGLIA AND MACROPHAGES IN THE BRAIN WITH ISCHEMIC AND TRAUMATIC INJURIES THAT CAUSE BBB BREAKDOWN

Severe damage to brains and spinal cords disrupts the BBB, resulting in massive infiltration of blood-borne cells, such as monocytes and neutrophils[9,22]. Discrimination of the roles of these immune cell types in pathophysiological processes is becoming possible, although further study is necessary[10]. Different responses of microglia and macrophages are seen in the ischemic lesion core and the ischemic penumbra or peri-ischemic region[11,27]. It should be noted that microglia are very vulnerable to various kinds of severe brain insults, including CO intoxication, as mentioned above[64]. Six hours after reperfusion in tMCAO (90 min-occlusion) in a rat model of severe stroke, microglia undergo apoptotic degeneration in the lesion core, whereas neurons appear unchanged[9,11]. Therefore, blood-borne macrophages and neutrophils are the main immune cells in the lesion cores and during the acute phase. However, microglia are both viable and become activated in the peri-region neighboring the core of the lesion and modulate pathological processes.

Roles of microglia

The activated microglia in the peri-ischemic regions bear large phagosomes that can be recognized by immunohistochemical staining of CD68[27]. Moreover, they frequently express NG2 chondroitin sulfate proteoglycan (NG2), which may be another marker for phagocytosing microglia and macrophages[27]. $CD68^+$-phagocytosing microglia in the SNr and GPi in the rat PD model brains also express NG2[47]. Such $NG2^+/CD68^+$ microglia engage in phagocytosis of degenerating neurons in the very limited region located along the border zone delineating the ischemic core and the penumbra[27]. The region is termed the demarcation zone[65] and is characterized by high expression of NG2. Neurodegeneration, known as delayed neuronal death, is still progressing in the zone in the subacute phase, which has long been a therapeutic target in stroke research to ameliorate the outcome of stroke.

Microglia are assumed to eliminate still viable neurons via phagocytosis in the ischemic penumbra[66,67]. Because of decreased blood flow, neurons reduce their ATP synthesis and, thus, frequently externalize phosphatidylserine (PS) on their surface. PS is a typical eat-me-signal molecule that is recognized by molecules, such as either Milk fat globule EGF-like factor 8 (MFG-E8) or protein S, which are, in turn, recognized by either vitronectin receptor or Mer receptor tyrosine kinase (MerTK) expressed by microglia. Expression of MFG-E8 and MerTK is enhanced either in activated microglia or in macrophages in the ischemic lesions. Knocking out expression of MerTK or MFG-E8 by microglia prevented the delayed neuronal loss considerably. These findings may indicate that $CD68^+/NG2^+$-phagocytosing microglia are aggravating cells in stroke pathology.

Administration of the CSF1R antagonist PLX3397 to mice depletes microglia. This pharmacological intervention was used to study the overall effects of microglia on the outcome of ischemic brain insults. Eliminating microglia increases infarct volume, indicating that the overall effects of microglia on the ischemic brain are ameliorative. Microglia in the non-ischemic regions may maintain the neuronal circuitry, suppress proinflammatory activation of astrocytes, and prevent infiltration by various leukocytes, such as T cells, monocytes, and granulocytes[68,69].

Activated microglia in the ischemic brain release a considerable quantity of transforming growth factor β1 (TGFβ1)[27], which is a strong immunosuppressive cytokine. Ischemic brain lesions should contain

abundant DAMPs, such as HMGB1[70] and peroxiredoxin[71], which are potential ligands for toll-like receptors (TLRs). DAMPs cause proinflammatory activation of microglia as lipopolysaccharide (LPS). However, expression of proinflammatory cytokine by both microglia and macrophages in the ischemic brain is not very remarkable[72]. TLR ligands strongly induce expression of inducible nitric oxide synthase (iNOS) by microglia in culture. However, microglia in the ischemic brain either do not express iNOS protein or do so very faintly. TGFβ1 expression increases gradually until 7 days after the ischemic insults. Either LPS or DAMPs induce phosphorylation of IκB kinase (IKK), causing degradation of IκB, enabling the major proinflammatory transcription factor NFκB to translocate into nuclei and resulting in increased transcription of mRNA for proinflammatory mediators[73]. However, after being incubated with TGFβ1 for ~24 h, primary cultured microglia do not respond to LPS treatments, even when the TGFβ1 is removed from the culture media. The TGFβ1-treated microglia cannot be classified into M2 polarized cells as they do not express M2 markers. TGFβ1 also inhibits phosphorylation of signal transducers and activators of transcription 1 (STAT1) and expression of interferon regulatory factor 1 (IRF1), both of which augment proinflammatory reactions of microglia in a sustained manner. When injected into the parenchyma of the ischemic brain, both microglia and macrophages lose their immunoreactivity to phosphorylated IKK (pIKK). Expression of other anti-inflammatory cytokines, such as IL-4, IL-10, or IL-13, is much weaker than is that of TGFβ1. These findings indicate that TGFβ1 is a key ameliorating factor released by both microglia and macrophages.

Roles of macrophages

Macrophages accumulate densely in the core of the ischemic and traumatic lesions where almost all microglia disappear[11,12,19,74]. Therefore, macrophages play central roles in the immunological modulation of pathophysiological processes in these lesions. As most of the accumulated macrophages express NG2[74], they have been called brain $Iba1^+/NG2^+$ cells (BINCs)[11]. At least some are involved in removing degenerated materials in the core. The macrophage-precursor monocytes invade inflamed brain tissues in ischemic and traumatic injuries through recognizing chemokines that are typically either CCL2 or monocyte chemoattractant protein 1 and either CX3CL1 or fractalkine[22]. Kinetic study in the rat tMCAO models showed that expression of CCL2 and CX3CL1 disappeared rapidly after tMCAO, within 2 days. When macrophage proliferation was inhibited by a single administration of the anti-cancer drug 5-fluorouracil 48 h after tMCAO, accumulation of macrophages at 7 dpr did not occur[12]. Therefore, proliferation of monocytes that infiltrate within 2 days of the onset of the events causes the massive accumulation of macrophages in the core of the lesion[11]. The reduction in number of the accumulated macrophages often causes death of the model rats. When BINCs were isolated from ischemic rat brains and transplanted into the core lesions of other stroke model rats, the BINCs proliferate hugely and the outcomes were ameliorated greatly. BINCs express various neuroprotective factors, such as IGF-1 and HGF, that may contribute to improved outcomes. Although BINCs were also found in lesions in aged human brains of stroke cases, however, the density was considerably less than that in the young rat model lesions[12]. This may be one reason why human stroke cases become more severe than do the young rat models. Overall, the collective effects of macrophages on ischemic brains appear to be ameliorative.

However, the effects of monocyte-derived macrophages may not be favorable during the acute phase. Microglia and macrophages were isolated individually from TBI model rats at 1.5 days after injury using a fluorescence-activated cell sorter based on the different levels of CD45 expression[10]. In this TBI model, oxidative injury may play a significant detrimental role in inducing neuronal degeneration, as an oxidative product 8-hydroxydeoxyguaine (8-OHdG) was found in neuronal nuclei. Reactive oxygen species (ROS) is the cause for this product, and most of the ROS may be derived from the mitochondria of macrophages[75]. Infiltrating macrophages produce much higher levels of mitochondrial ROS than do microglia. ROS is also produced by NADPH oxidase activity, and macrophages express much higher levels of NADPH oxidase than do microglia. Moreover, expression of IL-1β and iNOS is more significant in macrophages than in

microglia. Although NO is occasionally described as a neuroprotective factor[76], it can bind to superoxide O_2^- and form the highly toxic molecule peroxynitrite ($ONOO^-$)[77]. Conversely, microglia can save neurons from degeneration induced by oxidative stress[78,79]. Collectively, the roles of blood-borne macrophages are more detrimental than are those of microglia in the earliest phase of severe brain injuries.

Some different phenotypes of macrophages accumulate in the traumatic and ischemic lesion cores[10,19]. During the acute phase, 2 days after the onset of stroke, most macrophages exhibit a rather neurotoxic phenotype characterized by expression of IL-1β and iNOS and release of ROS[10]. However, most macrophages exhibit neuroprotective phenotypes characterized by expression of IGF-1 and HGF. This does not imply that a single population of macrophages turns into the two different (neuroprotective and neurodestructive) populations depending on the pathological processes. As the neuroprotective macrophages characteristically express NG2, they are termed BINCs. However, BINCs cannot be classified as M2-polarized macrophages; they express a typical M1 marker CD86 and not an M2 marker CD163, whereas they do not express IL-1β and iNOS in the ischemic lesion core. There is a minor subpopulation of $Iba1^+$/$CD68^+$ macrophages; though they do not express either NG2 or CD86, they express IL-1β, CCL2, and iNOS, in addition to TLR4, which may be the source of the proinflammatory factors. Characteristically, they express CD200[19,80], which mediates immunosuppressive signals primarily to myeloid cells expressing its receptor CD200R. Though the $CD200^+$/$NG2^-$ macrophages are likely the predominant population during the acute phase of severe brain injury, they undergo gradual apoptotic degeneration due to the oxidative injury, as evidenced by the accumulation of $8OHdG^+$ materials in their nuclei[10]. Importantly, as they are not proliferative, they decline in number along with the pathological processes[19]. Alternatively, BINCs may be a small population during the acute phase, but they are highly proliferative and become the main macrophage population in the lesion core. Figure 2 summarizes the two types of macrophages.

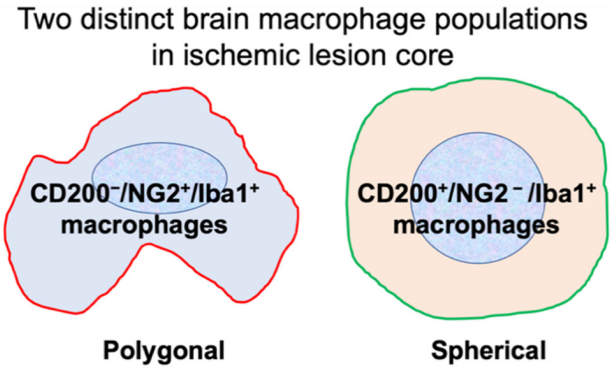
In conclusion, macrophages also play both favorable and unfavorable roles in brain pathology. However, unlike the case with microglia, there are different macrophage populations that exert favorable and unfavorable effects independently.

PHARMACOLOGICAL INTERVENTIONS

The term "double-edged sword" has been used frequently to describe the favorable and unfavorable effects of microglia and macrophages[81-83]. Pharmacological interventions have long been sought that would either induce or strengthen the favorable effects of these cells[15,84]. However, in the case of macrophages, it may be more likely that there are different subpopulations with different effects on neurons rather than there being opposing effects of a single population of macrophages. Nevertheless, numerous pharmacological interventions have been shown to modulate the natures of both microglia and macrophages. Some of these are discussed below. Here it is better to once again confirm the notion that the favorable and unfavorable phenotypes for neuronal survival are incompatible with the M1 and M2 classifications[18,19,72].

Glucocorticoids

Glucocorticoids have strong immunosuppressive effects on many kinds of cells and may be the most often-used anti-inflammatory agent in laboratory and clinical settings. Glucocorticoids may have stronger anti-inflammatory effects on microglia than do adrenergic agonists, minocycline, troglitazone, and antidepressants [Figure 3]. They cause microglial cells to shrink, reduce lysosomal enzyme activities, and suppress proliferation of primary cultured rat microglia[85]. They suppress expression of the proinflammatory mediators by LPS-treated cultured microglia primarily at the transcription level[43]. Glucocorticoids can bind not only to the glucocorticoid receptor but also to the mineralocorticoid receptor[85]. Mineralocorticoid receptor may mediate activating effects on microglia rather than inhibitory effects. As the major rodent glucocorticoid corticosterone has an affinity for both mineralocorticoid



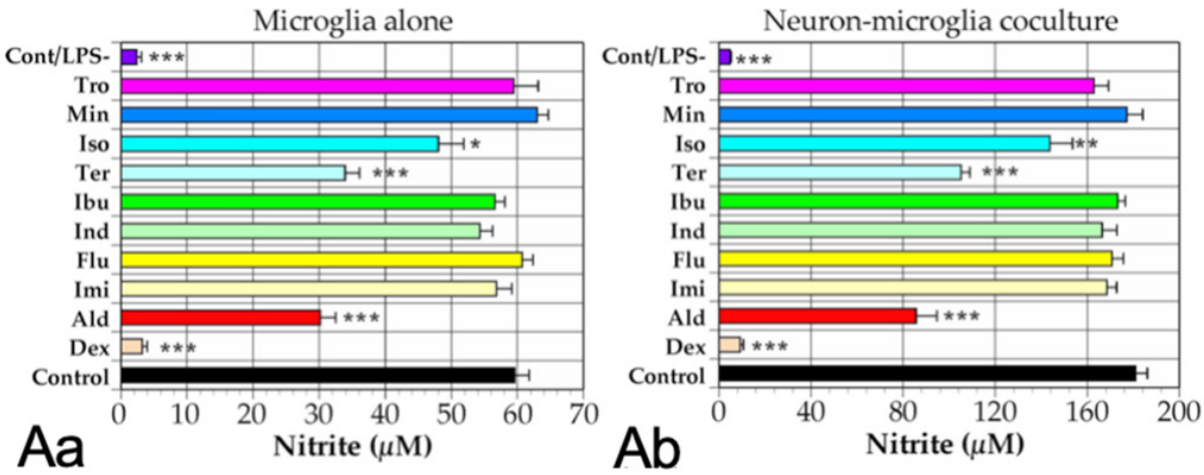
Antigen expression	NG2⁺ macrophages	NG2⁻ macrophages
NG2	+	–
CD200	–	+
CD8	–	+
TLR4	–	+
CD86, M1 marker	+	–
CD163, M2 marker	–	+
Ki67	+ (56%)	– (5%)
TLR4	–	+
CCL2	–	+
IL-1β	–	+
iNOS	– (3%)	+ (50%)
TGFβ1	–	+

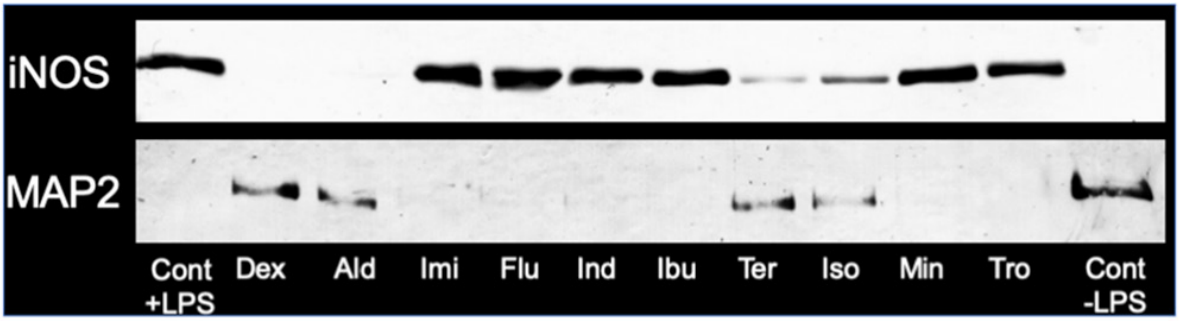
Figure 2. Distinctions between two populations of blood-borne macrophages accumulated in ischemic core lesions of a rat stroke model prepared by tMCAO. $CD200^-/NG2^+/Iba1^+$ macrophages (BINCs) are the dominant populations occupying the most ischemic core regions. The two populations cannot be classified into M1 and M2 categories. The data come mainly from the data Matsumoto *et al*.[19] (2015)

receptor and glucocorticoid receptor, it exerts biphasic effects on the microglial proinflammatory actions - activation at low concentrations and inhibition at high concentrations.

A synthetic glucocorticoid dexamethasone (Dex), a specific ligand for glucocorticoid receptor, strongly inhibits LPS-induced NO release and expression of mRNA for IL-1β and TNFα[43]. When primary cortical neurons were cocultured with microglia in the presence of LPS, neurons were degenerated. As this LPS-induced neurodegeneration was prevented by an NOS inhibitor, L-NMMA, the neurodegeneration is caused by NO derived from activated microglia. However, Dex, rather than the NOS inhibitor, suppresses the release of NO more strongly and prevents the degeneration almost completely [Figure 3]. Moreover, Dex increases the expression of mRNA for the neuroprotective factors HGF and IGF-1. The findings indicate that glucocorticoids can induce a neuroprotective microglia phenotype even in the presence of LPS in culture or of DAMPs in the damaged brain parenchyma.

Because of such favorable effects, glucocorticoids have been studied in various animal brain disease models with neuroinflammation. As mentioned, in the rat 6-OHDA-induced PD model, microglia in the SNc are activated in response to degeneration of DA neurons. Chronic administration of glucocorticoids appears to inhibit microglia activation and enhance both the viability of DA neurons and the amount of tyrosine hydroxylase, a rate-limiting enzyme for DA synthesis [Figure 3]. However, glucocorticoids have several adverse effects, including induction of osteoporosis, impaired glucose tolerance, and increased susceptibility to infection. Moreover, glucocorticoids potentially impair cognitive functions by damaging the circuitry of the hippocampus, in which glucocorticoid receptor is expressed abundantly[86,87]. Nevertheless, as shown in Figure 3, chronic oral administration of glucocorticoid at a rather low dose of


Microglia alone
Cont/LPS-
Tro
Min
Iso
Ter
Ibu
Ind
Flu
Imi
Ald
Dex
Control
0 10 20 30 40 50 60 70
Nitrite (μM)
Aa
Neuron-microglia coculture
0 40 80 120 160 200
Nitrite (μM)
Ab


iNOS
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Iso
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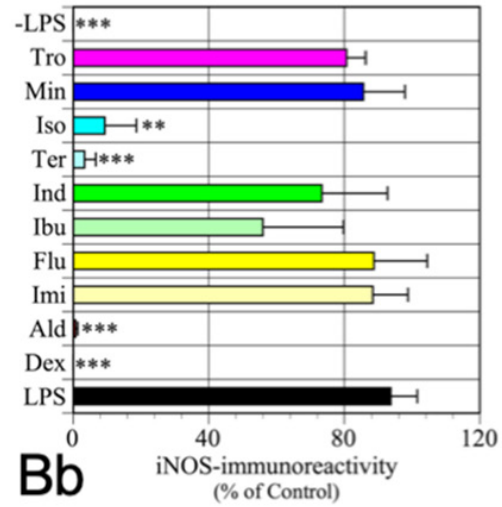
Cont -LPS


-LPS
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0 40 80 120
iNOS-immunoreactivity
(% of Control)
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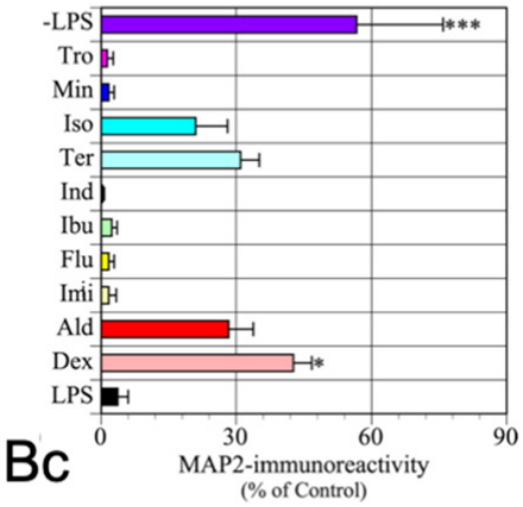
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LPS
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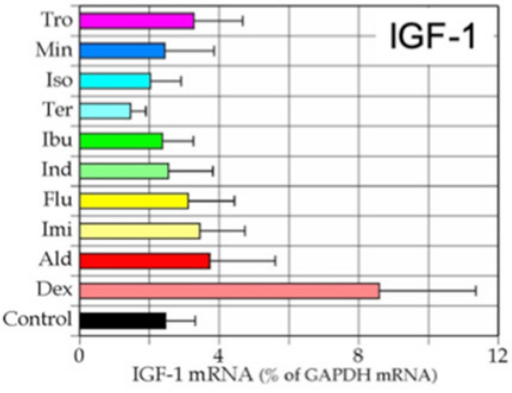
MAP2-immunoreactivity
(% of Control)
Bc


IGF-1
Tro
Min
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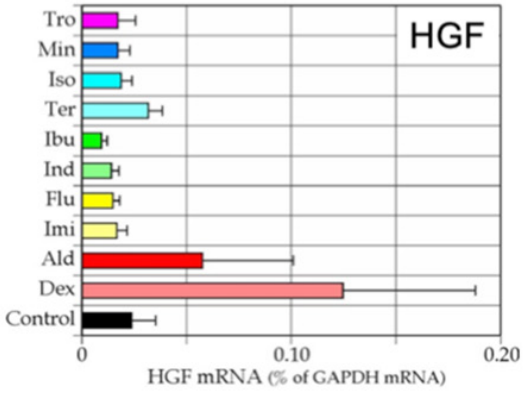
Imi
Ald
Dex
Control
0 4 8 12
IGF-1 mRNA (% of GAPDH mRNA)


HGF
Tro
Min
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Ter
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Ind
Flu
Imi
Ald
Dex
Control
0 0.10 0.20
HGF mRNA (% of GAPDH mRNA)

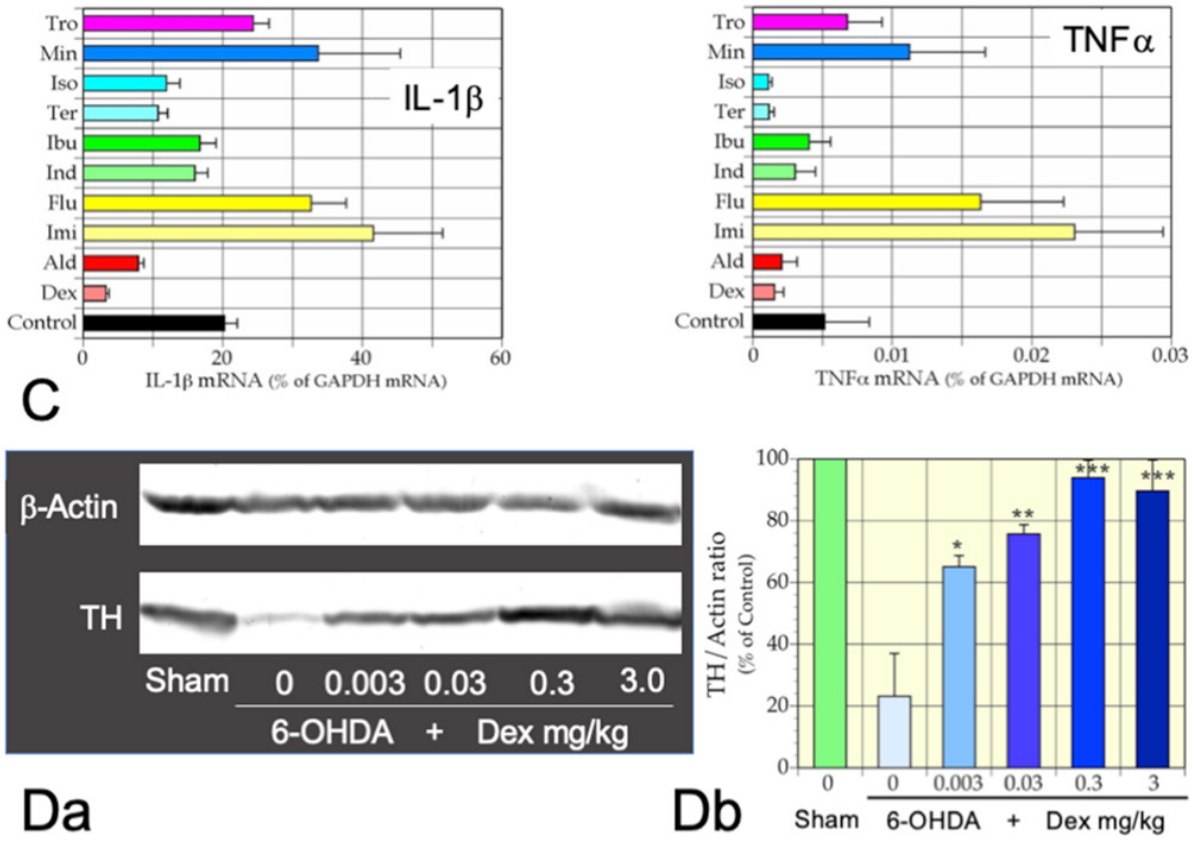

Figure 3. Immunomodulatory effects of a synthetic glucocorticoid Dex on microglia. A: effects of 10 agents on NO release by rat primary microglia (A-a) and rat primary cortical neuron-microglia coculture (A-b) incubated for 48 h with LPS. Nitrite levels in conditioned media were determined. Dex suppressed NO release most effectively. *P < 0.05, **P < 0.01, ***P < 0.001, respectively *vs*. LPS. B: effects of LPS and the agents on LPS-treated neuron-microglia cocultures. B-a: representative immunoblots showing the contents of MAP2 and iNOS proteins in the cocultures. (-LPS) denotes an absence of LPS; B-b: statistical comparison of suppressive effects on LPS-induced iNOS expression; B-c: neuroprotective effects of the 10 agents on the LPS-treated coculture. Strong iNOS expression was correlated with loss of MAP2-immunoreactivity. Only Dex protected neurons significantly from microglial neurotoxicity. Data from four independent cultures are expressed as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, respectively, *vs*. LPS. C: microglia were incubated with an indicated agent for 16 h in the absence of LPS. Only Dex increased expression of mRNAs encoding IGF-1 and HGF significantly while suppressing expression of IL-1β and TNFα mRNAs. n = 4, *P < 0.05, **P < 0.01, ***P < 0.001, respectively, *vs*. control. D: dose-dependent ameliorative effects of Dex on a rat 6-OHDA-induced PD model. D-a: representative immunoblots showing dose-dependent effects of Dex on the content of TH, a DA neuron marker; D-b: statistical analyses of the dose-dependent effects of Dex. A dose 0.003 μg/kg weight of Dex significantly prevented the reduction in TH immunoreactivity. Data from four rats for each dose were expressed as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, respectively *vs*. 6-OHDA/Dex 0. The data are from unpublished ones by Wada *et al*. (unpublished data). Dex: dexamethasone; Ald: aldosterone; Imi: imipramine; Flu: fluvoxamine; Ind: indomethacin; Ibu: ibuprofen; Ter: terbutaline; Iso: isoproterenol; Min: minocycline; Tro: troglitazone; LPS: lipopolysaccharide; TH: tyrosine hydroxylase; DA: dopaminergic; PD: Parkinson's disease; HGF: hepatocyte growth factor; IGF-1: insulin-like growth factor 1; 6-OHDA: 6-hydorxydopamine; iNOS: inducible nitric oxide synthase

3 μg/kg body weight for 6-OHDA-induced PD model rats prevented the reduction in tyrosine hydroxylase immunoreactivity in the SNc to a significant extent. It may be worth administering this dose in a clinical setting.

As mentioned above, microglia play a favorable role by eliminating hyperactive glutamatergic synapses from STN in the PD pathophysiology[47]. When 6-OHDA-induced PD model rats are administered a high dose of glucocorticoid, the motor deficits are aggravated. This may be one example demonstrating that strong immunosuppressive interventions for activated microglia and macrophages have an aggravating effect, as they have both favorable and unfavorable roles.

Noradrenaline and other cAMP-elevating agents

As shown in Figure 3, noradrenaline (NA) and related agents have significant inhibitory effects on activated microglia. These effects have been attributed mainly to the adrenergic β2 receptor that increases levels of intracellular cAMP[73,88]. The β2 agonist terbutaline strongly suppressed LPS-induced proinflammatory activation of microglia. cAMP-elevating agents, such as phosphodiesterase (PDE) inhibitors also exhibited strong inhibitory effects on LPS-treated microglia[89]. Rolipram, an inhibitor of the cAMP-selective hydrolase PDE4, may be the PDE inhibitor with the strongest inhibitory effects on rat primary microglia. A cAMP-analogue, 8-bromo cAMP, and forskolin, an activator for adenylate cyclase, also have strong inhibitory effects on microglia *in vitro*. Furthermore, the cAMP-elevating agents inhibit proliferation of microglia *in vitro*[90]. However, it should be noted that adrenergic α1 receptor agonists such as phenylephrine can inhibit microglial activation to an extent similar to that of terbutaline[73,88]. The inhibitory effects of NA cannot be eliminated by an inhibitor for cAMP-dependent protein kinase[73]. NA, α1, and β2 agonists strongly prevent LPS-induced translocation into the nuclei of NFκB[73]. This effect is mediated mainly by suppression of IκB degradation. NA and the agonists suppressed LPS-induced phosphorylation of STAT1 and expression of IRF1. IRF1 may contribute significantly to microglia activation[43]. However, the precise molecular mechanisms underlying the inhibitory effects of NA, in addition to the agonists, remain to be identified.

These studies may indicate that BBB-permeable adrenergic agonists are promising agents for suppressing neuroinflammatory processes in pathologic brains. The curative effects of BBB-permeable β2 agonists, while preventing the proinflammatory nature of microglia in the SNc, have been shown in murine PD models[91]. In AD pathology, NA neurons in the locus ceruleus, which is the most important NA source in the brain, undergo degeneration leading to microglia activation. This subsequently results in further neuronal degeneration[92]. However, there are many conflicting studies demonstrating the stimulatory actions of NA and the adrenergic agonists on microglia in the brain[93].

TGFβ1

TGFβ1 may be the most abundantly released cytokine from microglia and macrophages in severely damaged brains[27,72]. TGFβ1 suppresses iNOS expression by LPS-treated primary rat microglia almost completely in culture at both mRNA and protein levels. The inhibitory effect of TGFβ1 is as strong as is 100 nM of Dex in culture experiments[72]. As mentioned above, once incubated with TGFβ1, LPS cannot induce NFκB translocation into nuclei in microglia. TGFβ1 also inhibits LPS-induced phosphorylation of STAT1 and expression of IRF1. Although TGFβ1 does not increase expression of the M2 markers CD206 and Ym1, it increases expression of the neuroprotective factors HGF and bFGF. When TGFβ1 was injected into ischemic tissues 48 h after MCAO, the immunoreactivity of pIKK in the tissue surrounding the injection site was suppressed markedly in a dose-dependent manner.

Severely damaged brain tissues contain DAMPs that should induce proinflammatory activation of microglia and macrophages. DAMPs bind to TLRs on both microglia and macrophages, resulting in translocation of NFκB into nuclei. Microglia and macrophages should then display proinflammatory phenotypes expressing proinflammatory mediators. However, there were very few pIKK-bearing or iNOS-expressing microglia/macrophages in the lesion, especially during the subacute phase when TGFβ1 expression is high. Interventions to increase the actions of TGFβ1 for stroke models have been shown to ameliorate the outcomes for severely damaged brains[94]. Thus, TGFβ1 may ameliorate severe CNS damage through inducing anti-inflammatory phenotypes of microglia and macrophages.

Bromovalerylurea

Bromovalerylurea (BU; $C_6H_{11}BrN_2O_2$, CAS: 496-67-3) is a hypnotic/sedative that was developed more than a century ago[95]. It is not prescribed currently because of its weak actions as a hypnotic/sedative compared

with newer agents such as benzodiazepines and also due to dependency. Recently, it has been found to have strong anti-inflammatory effects[10,43,96,97]. The effects of BU on LPS-treated primary rat microglia are as strong as those of Dex[43]. Like Dex, BU increases the expression of neuroprotective factors such as HGF or IGF-1 by LPS-treated primary microglia while inhibiting the expression both of proinflammatory factors and of the proinflammatory transcription factors IRF1, IRF7, and IRF8[43,98]. Although BU does not suppress LPS-induced NFκB translocation into nuclei, its inhibitory effects are exerted at the transcriptional level. BU inhibited ATP synthesis in mitochondria; this may be related to its anti-inflammatory effects[10,96]. Moreover, BU inhibits Janus kinase 1 activity, thus suppressing the phosphorylation of STAT1 and the subsequent expression of IRF1[43].

BU prevented the death of rats with cecum ligation and puncture-induced sepsis by suppressing the proinflammatory activation of peritoneal macrophages[97]. Oral administration of BU ameliorated PD in rat models while inhibiting the expression of proinflammatory mediators in the ventral midbrain[43]. When BU is added to the LPS-treated microglia/neuron coculture, it can almost completely inhibit neuronal death by almost completely suppressing the release of NO. In rat TBI models, BU ameliorated neurological deficits considerably while inhibiting expression of chemokine CCL2 and suppressed monocyte infiltration of the lesion[10]. Furthermore, BU inhibited mitochondrial ROS release by macrophages strongly. On the other hand, BU did not affect the accumulatio of favorable macrophages at later time points.

As BU is a hypnotic/sedative, it crosses the BBB easily. It may hold promise as an agent for ameliorating a range of brain diseases and injuries. However, BU is associated with marked dependency, and it has been used for suicide. Nevertheless, recent research has shown that the sleep-inducing effects of BU necessitate a higher dose than that required to produce the anti-inflammatory effects. As a hypnotic, BU increases the total sleeping period while reducing rapid eye movement sleep at a dose of 250 mg/kg for rats (Takeda *et al*., unpublished observation). Conversely, BU has been administered to sepsis, TBI, and PD model rats at a dose of 50 mg/kg. The findings suggest that BU could be used as a specific anti-inflammatory drug without significant dependency.

Agents that elevate expression of antiapoptotic factors in neurons

Microglia and macrophages can be activated by the presence of damaged neurons because of released DAMPs. Moreover, damaged neurons will reduce the activity of flippase, which is required for asymmetric distribution of phospholipids. Flippase reverses the translocation of PS to the exoplasmic face of the plasma membrane in an ATP-dependent manner. Therefore, even weak neuron damage that reduces intracellular ATP results in translocation of PS to the exoplasmic surface of the plasma membrane. The PS will be recognized by proteins such as MFG-E8 and also by complement C1[99]. These changes stimulate phagocytic elimination of neurons by microglia and macrophages in a process called phagoptosis[66]. Therefore, either suppression of the apoptotic changes or mitochondrial damage will suppress the unfavorable activation of microglia and macrophages[100,101].

A hematopoietic cytokine IL-3 has been shown to increase Bcl-xL expression in neurons in ischemic hippocampi[102] and in the SNc of PD model rats[44]. Moreover, GM-CSF also increases Bcl-xL expression[103]. Simultaneous administration of IL-3 and GM-CSF shows more marked ameliorative effects in TBI and PD model rats. The mixture of the cytokines also inhibited neuronal loss in a rat stroke model prepared by MCAO. Subcutaneous administration of the cytokine mixture to PD model rats inhibited IL-1β and TNFα expression in the ventral midbrain, whereas the expression of IGF-1 and HGF was increased. The favorable changes in microglia may be mediated by the ameliorated survival of neurons. The cytokines also have specific direct effects on microglia and macrophages[13,104]. Addition of cytokines to the culture of isolated macrophages (BINCs) from TBI lesions increased HGF and IGF-1 expression, whereas it did not affect IL-1β expression[13]. This indicates that inhibition of the proinflammatory nature of the cytokines may be

attributed to ameliorated neuronal survival rather than the direct effects on macrophages and microglia. Thus, an agent that can increase expression of anti-apoptotic factors in neurons would increase the number of favorable microglia and macrophages.

Ginsenosides are natural products isolated from the plant ginseng. Among the ginsenosides, ginsenoside Rb1 and its derivatives strongly ameliorate the outcome of stroke[105] and TBI[106] models, while increasing Bcl-xl expression. Along with their direct effects on neurons, ginsenosides have been shown to inhibit the proinflammatory activation of microglia. It remains to be determined whether they inhibit LPS-induced proinflammatory activation of microglia and macrophages[107,108].

CONCLUSION

Microglia and macrophages have profound effects on the pathophysiological processes of several brain pathologies. They become activated in response to pathological changes of neurons that produce DAMPs and express PS on their surface. Microglia and macrophages can have ameliorative and/or deleterious effects on the CNS depending on the severity of the disease or injuries, time course, and BBB disruption. Even within the same pathology, the cells exert both different effects in completely different ways, as described in the cases of PD and the spinal cord of the peripheral nerve injury model. The development of pharmacological interventions to regulate the response of microglia and macrophages has long been anticipated. However much more research into their responses is required before that goal can be attained.

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Authors' contributions

The author contributed solely to the article.

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Conflicts of interest

The author declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

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Review

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Bilirubin and inflammation in neurodegenerative and other neurological diseases

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Abstract

Inflammation links neurodegenerative, neuropsychiatric and other neurological diseases (NDs) with acute brain events. It is responsible for the alteration of neurotransmission and circuity, brain architecture, and cell fate, affecting mood and personality (anxiety, depression and schizophrenia) and behavior (decline in cognitive, motor and speech abilities, altered sleep, fatigue, pain sensitivity and dementia). Inflammation is also a key component in systemic chronic diseases (cardiovascular disease, cancer, diabetes, and metabolic syndrome), in which bilirubin has been demonstrated to improve the diseases by acting as a multi-target antiinflammatory molecule, and where the evaluation of pharmacological modulation of the pigment level as a therapeutic approach has already started. While altered serum bilirubin levels have been reported in ND patients, the potential activity of bilirubin in the brain is vague. This review summarizes the available fragmentary information on the interplay of bilirubin with neuroinflammation, aiming to elucidate the pigment's role in the central nervous system environment.

Keywords: Neuroinflammatory diseases, bilirubin, brain, heme oxygenase, biliverdin reductase, inflammation, homeostasis

INTRODUCTION

Bilirubin (unconjugated - UCB) is the final metabolite of hemoglobin, which is processed in the liver (by conjugation to 1 or 2 molecules of glucuronic acid - CB: conjugated bilirubin) before

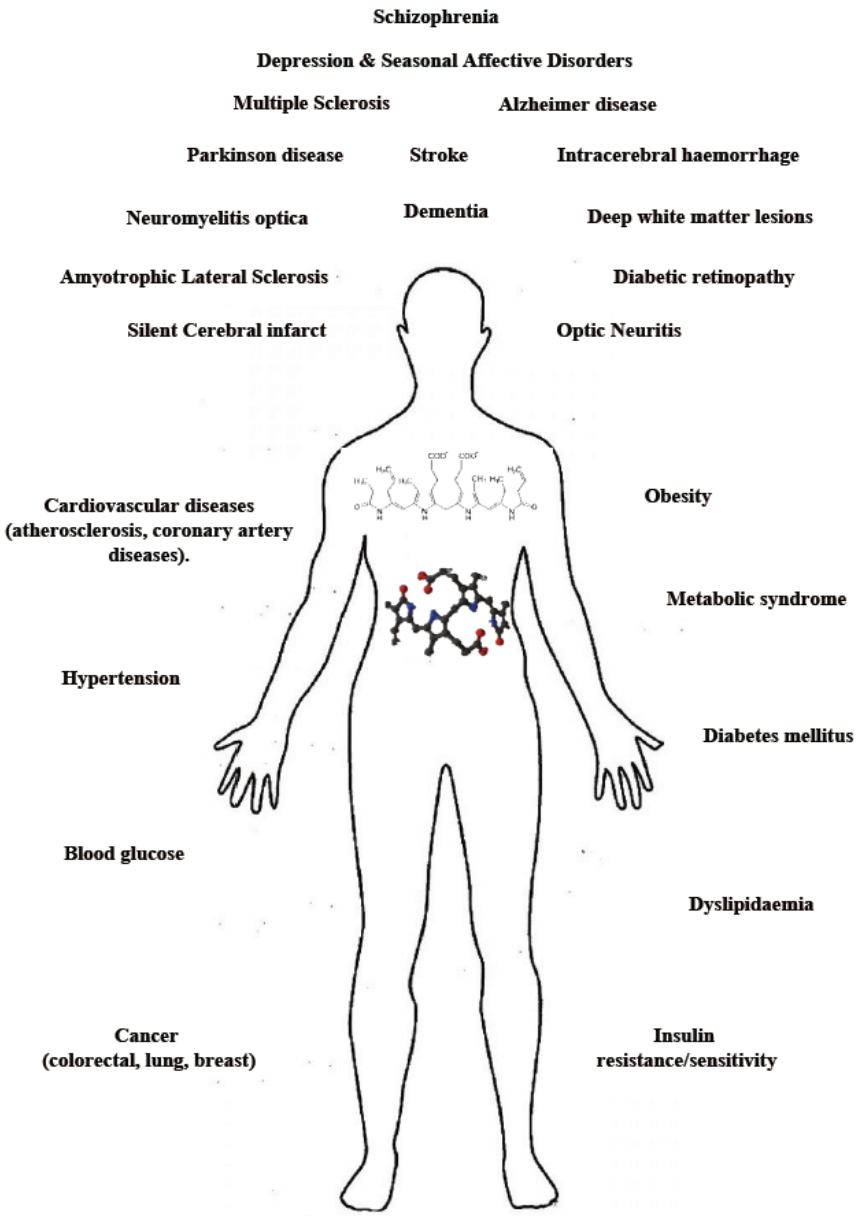

Figure 1. Bilirubin & pathological conditions. Representation of the known CNS and not-CNS diseases where TSB is altered. For references of CNS-diseases, see text. For details on not-CNS diseases, see references (according to Gazzin *et al*.[2] and Wagner *et al*.[3])

elimination through urine and feces. Increased level of bilirubin (UCB and CB) in the blood is a well-recognized marker of hepatic damage. Recently, slightly elevated serum bilirubin concentration emerged as a biomarker of resistance versus chronic diseases[1]. Epidemiological data have revealed a reduced prevalence of type 2 diabetes, obesity and metabolic syndrome, certain cancers, and especially cardiovascular diseases and related causes of mortality in Gilbert's syndrome patients, showing mild hyperbilirubinemia[2,3] [Figure 1]. The effect of the pigment has been demonstrated to be due mainly to the unconjugated (or indirect) moiety of bilirubin (UCB). UCB, especially in its free form [free bilirubin (Bf), the portion of UCB exceeding the serum albumin binding capacity], enters tissues from blood, acting as a powerful antioxidant molecule at nanomolar concentrations, where it is able to counteract 10,000 times higher levels of hydrogen peroxide (H_2O_2)[4]. This capability has been related to the UCB-biliverdin cycle [Figure 2], which is able to regenerate the pigment consumed by oxidants and acts complementary to cellular glutathione (GSH)[4-6]. In the last years,

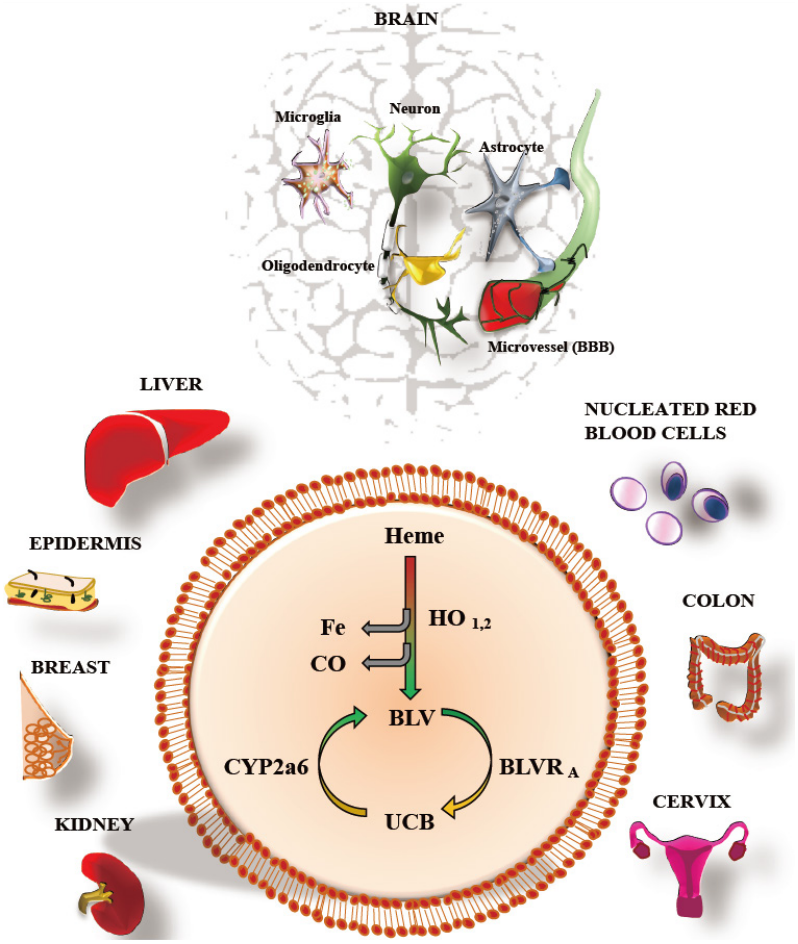

Figure 2. The yellow players. Heme: Haemoglobin; HO: Heme oxygenase; Fe: Iron; Co: Carbon monoxide; BLV: Biliverdin; BLVR: Biliverdin-reductase; UCB: Unconjugated bilirubin (indirect bilirubin); CYP: Cytochrome oxygenase. The picture shows the "yellow players" (see text) and the tissues able to produce *de novo* bilirubin, based on the cited literature (Takeda *et al*.[7])

a more complex and fascinating scenario has emerged. First, various cells have been demonstrated to possess the complete enzymatic apparatus necessary for producing UCB themselves [Figure 2][7,8]. A more expanded and intricate view has emerged from the discovery of the interplay of UCB (and the enzymes involved in its production and recycling, altogether called the "yellow players") with cellular functions, signaling pathways, and defense/adaptation mechanisms (not restricted to redox state). Collectively, these findings suggest a greater role for the yellow players in cellular homeostasis and defense against diseases[2,3]. The interplay of the yellow players with neurological and neurodegenerative diseases is still much less explored. In this article, we review the currently available evidence on the potential roles of bilirubin and other yellow players in neurological disease, with special emphasis on inflammation. We will also address the role of enzyme modulation in bilirubin metabolism, with the goal of increasing the systemic level of bilirubin and the protection it confers.

INFLAMMATION AND NEUROLOGICAL DISEASES: AN OVERVIEW

Chronic neurodegenerative pathologies are currently the most dominant clinical conditions. They comprise multiple sclerosis (MS), amyotrophic lateral sclerosis (ALS), Parkinson's disease (PD), Alzheimer's disease (AD) and vascular dementia. Consistent data show that the inflammatory process can be triggered by protein misfolding or protein accumulation, which are the initial events of a given pathology (i.e., amyloid for AD, tau protein for frontal dementia, alpha synuclein for PD, *etc.*)[9].

Aging has been associated with a low-grade sterile inflammatory status of the immune system, in which interleukin-6 (IL6), IL1β and tumor necrosis factor (TNF) are key players, more evident in an unhealthy state. Neuroinflammation can be considered one of the most important etiological factors in age-related neurodegeneration[10], associated with a reduction in neuron number, a decrease in neuronal arborization, and loss of spines[11]. What fits particularly in this theory, is the strong evidence that in the aging brain, both macrophages and microglia react with a prolonged and overactive response to stimuli[12]. This overactivation induces the production of reactive oxygen species and attracts peripheral leukocytes, and both these conditions can activate glial cells[13]. The activation of glial cells promotes telomere shortening, which can be a contributor in different neurological conditions, such as AD[14]. Resulting impaired phagocytosis alters the removal of toxic compounds, such as amyloid-beta (Aβ) and alpha-synuclein (αSyn)[15]. Microglial activation is believed to be involved in the occurrence of deterioration in various neurodegenerative diseases, such as AD, PD, ALS, and MS[15]. Microglia are the main immune cells of the central nervous system (CNS), and as the first line of defense, microglia play an important role in the inflammatory reaction[16]. In AD, microglia are known for their role in cleaning up Aβ. Meanwhile, in PD, a pathological αSyn aggregation can induce microglial activation and dysfunction[17]. Microglia are also directly involved in MS by producing cytokines such as tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ), superoxide (O^{2-}) or nitric oxide (NO), and release of proteolytic and lipolytic enzymes, which can destroy the myelin sheath[15,18].

Neuroinflammation and mitochondrial dysfunction are common features of chronic neurodegenerative diseases. Both conditions can lead to increased oxidative stress, which leads to excess reactive oxygen species and reactive nitrogen species, resulting in a cascade of events, with injury to polarized bilayers, lipid peroxidation, lysosomal intrinsic activity and autophagy; all these events together, permit self-potentiation of an inflammatory cascade[19].

Inflammation also plays a role in cerebral small-vessel disease (cSVD), which usually manifests as stroke, cognitive impairment, dementia, physical disability and depression[20]. Inflammation in cSVD might be explained by the role of endothelial cells in the blood-brain barrier. Endothelial cells communicate with pericytes, astrocytes, microglia, and neural stem cells in the neurovascular unit. Damage to CNS tissues leads to the disruption of tight junctions that link endothelial cells[21]. It is followed by the infiltration of neutrophils and monocytes, the activation of microglia and astrocytes and the invasion of T and B cells. This inflammatory response is mediated by the nuclear factor κ-light-chain-enhancer of activated B cells (NFκB) pathway and strongly associated with neuroinflammation in the acute phase of various vascular injuries such as stroke, diabetic retinopathy, and AD[22,23].

Bilirubin has been reported to act on all the above-mentioned molecular mechanisms. The following paragraphs review the state-of-the-art of what it is known about bilirubin and the brain.

BILIRUBIN: A MARKER FOR NEUROLOGICAL DISEASES

Cumulative clinical evidence demonstrates the alteration of total serum bilirubin (TSB) level in neuroinflammatory diseases, including schizophrenia, MS, PD, AD, ALS, stroke, diabetic retinopathy, *etc.* (for details, Table 1). For obvious reasons (limited CNS sample availability, especially in the early stages of diseases), few data on the potential molecular role of the pigment in these pathologies are available, and cause-effect studies are possible only by using experimental models.

Schizoprenia

Multiple contrasting studies exist on the potential correlation between the serum level of UCB and schizophrenia. Studies have reported an increase in UCB level, as in Gilbert's syndrome in schizophrenia compared to other psychiatric diseases (affective disorder and neuropsychosis) and healthy controls[24].

Table 1. TSB level in neurological diseases

Dis.	Study design	# Subjects Dis/Ctrl	TSB Dis/Ctrl	CB Dis/Ctrl	UCB Dis/Ctrl	*P*	Ref.
AD	CC	101/101	10.26/15.39			< 0.001	[119]
AD	CC	pAD: 12/MC-AD: 12/HS: 12	4.62/3.93/12.48			< 0.05	[120]
ALS	CC	Short duration: 19/ long duration: 11	12.31/7.70			0.011	[115]
DR	CC	67/diabetic no DR: 35	10.1/15.1			< 0.01	[84]
DEM	CS /	Cogn Imp: 31/no Cogn Imp: 33	11.3/13.5			0.020	[117]
	CC	Cog. Imp. 31/40	11.3/15.0			0.003	
MS	CC	133/88	11.08/16.47	2.20/4.26	8.88/2.2	0.001	[87]
NMO	CC	67/98	12.25/16.15		9.17/13.22	< 0.001	[80]
NO	CC	42/48	11.8/15.5	2.7/4.1	8.7/11.4	< 0.01	[79]
PD	CC	420/435	9.57/7.70			< 0.001	[45]
PD	CS/CC	drug naïve PD: 75/75	12.65/8.72			< 0.001	[46]
PD	CC	LD-PD: 162/untr-PD: 93/ HS: 224	12.31/10.94/10.26			< 0.001	[48]
PD	CC	425/460	12.09/12.3	3.73/3.15	8.36/9.15	<0.05	[47]
SCI	CC	343/2522	8.3/10.7			< 0.001	[88]
SCZ	CC	34/114	9.74/11.14			0.04	[41]
SCZ	CC	72/65	6.5/12.2			10^{-13}	[42]
SCZ *vs.* BD	CC	71/BD: 69	10.62/8.04*		7.87/5.47**	*0.02 **0.03	[26]
SCZ *vs.* BD	CS	50/BD: 43	8.89/7.01[a]		6.84/4.45[b]	[a]0.027 [b]0.004	[27]
SCZ *vs.* BD	CS	44/BD: 56			7.01/4.95	< 0.0001	[25]
SCZ *vs.* SAD	CS	44/SAD: 99			7.01/5.81	< 0.03	

DDis: specification of the disease; Ctrl: specification of the controls or group of comparison (for detail see each study). When unspecified = healthy population; TSB: total serum bilirubin (μmol/L); CB: conjugated bilirubin or direct bilirubin (DB, μmol/L); UCB: unconjugated bilirubin or indirect bilirubin (IB, μmol/L); AD: alzheimer disease; ALS: amyotrophic lateral sclerosis; BD: bipolar disorders; CIS: clinically isolated syndrome; DEM: dementia, Cogn.Imp: subject with DEM and cognitive impairment; No Cogn.Imp: subject with DEM but not cognitive impairment; DR: diabetic retinopathy; HS: healthy subjects; MC-AD: mild cognitive Alzheimer disease; MS: multiple sclerosis; NO: neuritis optica; NMO: neuromyelitis optica; pAD: probable Alzheimer disease; PD: parkinson disease; LD-PD: L-Dopa treated PD patients; Untr.PD: untreated PD patients; SAD: schizoaffective disorder; SCI: silent cerebral infarct; SCZ: schizophrenia; CC: case control; CS: cross-sectional. *,[a]*P* value for TSB, **,[b]*P* value for UCB

More interestingly, after antipsychotic treatment, UCB decreases in 80% of subjects. Gama Marques *et al.*[25] also reported that mean UCB levels are clearly higher in patients with schizophrenia than patients with schizoaffective disorder and bipolar disorder. Additionally, retrospective and recent prospective studies indicate that serum UCB level in patients with schizophrenia is higher than those with bipolar affective disorder (1.40- and 1.53-fold, respectively)[26,27]. Notably, all these studies included only subjects with normal liver enzymes and no pre-existing liver disease or other confounding factors, further emphasizing the pivotal role of UCB increase. UCB can be viewed as a potential biomarker to distinguish schizophrenia from other psychiatric disorders[28].

UCB level also shows a correlation with schizophrenia symptoms. Patients with hyperbilirubinemia have shown significantly higher scores on the positive and general psychiatric subscales of the PANSS (Positive and Negative Syndrome Scale)[24]. In concordance with the previous finding, UCB elevation has been found frequently in psychotic episodes of schizophrenia[25,29]. Radhakrishnan *et al.*[26] reported that UCB levels are higher in paranoid schizophrenia than non-paranoid schizophrenia. Meanwhile, a recent study by Pradeep *et al.*[27] found no association between serum UCB levels with the severity of psychopathology in schizophrenia subjects.

Schizophrenic subjects with unconjugated hyperbilirubinemia show a significant enlargement of cerebral ventricles[30] and abnormalities of brain metabolism compared to both normobilirubinemic schizophrenia patients and normal controls[31]. Neuroinflammation in schizophrenia is characterized by increased serum concentrations of proinflammatory cytokines, including IL1β, IL6, and transforming growth factor-β

(TGFβ) and microglial activation[32,33]. This inflammatory picture is similar to that observed in kernicterus spectrum disorders, the neurological sequel due to severe neonatal hyperbilirubinemia[34]. Dalman and Cullberg[35] reported that neonates experiencing severe neonatal hyperbilirubinemia (TSB > 15 mg/dL) might later have an increased frequency of mental disorders. This clinical hypothesis is supported by the finding in the Gunn rat, the animal model for severe neonatal hyperbilirubinemia[36], showing severe hyperbilirubinemia in the first weeks of life[37]. The Gunn rat displays a stereotypical behaviour, a supposedly typical symptom of schizophrenia, associated with microglia activation (indicative of inflammation), decreased ribosomal protein synthesis activity in neuronal cells, decreased neurogenesis, and increased apoptosis[38,39].

On the other hand, multiple studies have reported lower TSB levels among schizophrenia patients compared to patients suffering from some other psychiatric disorder and healthy controls[40-42]. To reconcile these contradictory findings, Vítek *et al.*[42] evaluated the correlation of bilirubin level with variability of the promoter of the gene for *UGT1A1* (uridine-diphospho-glucuronosyltransferase 1A1) (responsible for hepatic conjugation of UCB) between schizophrenia patients and controls. They noticed that an increase in bilirubin of 1 μM (0.06 mg/dL) could reduce the odds for schizophrenia status up to 19%.

Notably, UGTs participate in the metabolism of dopamine[26], a critical neurotransmitter, the loss of which is responsible for motor PD, and dopaminergic over activity has recently been suspected responsible for psychosis in schizophrenia[43]. An additional potential link between these two pathologies might be heme oxygenase-1 (HO-1). The overexpression of this enzyme in the astrocytes of glial fibrillary acidic protein-heme oxygenase-1 (GFAP-HMOX1) transgenic mice resulted in increased subcortical oxidative imbalance with the induction of mitochondrial damage and autophagy, augmented dopamine and serotonin levels in the basal ganglia, and reduced dopamine 1 receptor (D1) in the nucleus accumbens, and enzyme overexpression caused degeneration of axons in the hippocampus and hyperkinetic behavior[44]. However, there is still no clear evidence of HO-1 alteration in the clinical setting for schizophrenia.

PD

PD is another neurological disease linked to UCB. A study comparing 420 PD patients and 435 healthy control showed not only a significant increase in bilirubin in PD patients but also a negative correlation between bilirubin level and progression from a less to more severe staging of the disease[45]. Complementary with this study, Moccia *et al.*[46] also reported higher TSB concentration in drug-naive PD patients compared to controls, and Qin *et al.*[47] also observed an upregulation of levels of direct bilirubin (conjugated bilirubin, in clinical terminology), accompanied by reduced UCB (indirect bilirubin) levels in PD patients compared to the healthy group. Notably, L-DOPA treatment (the most used therapeutic approach to PD, able to improve symptomatology temporarily), has been found to increase TSB by about 20%[48]. Because L-DOPA may increase the oxidative stress causing dopaminergic neuron loss in PD, and since bilirubin is a well-known antioxidant, an increased bilirubin level has been interpreted as a possible protective response to the disease[48].

As noted in the Introduction, almost every cell in the body possesses the full enzymatic equipment for producing UCB itself[7] [Figure 2]. Modulation of the enzymes responsible for UCB production in the brain of subjects with neurological/neurodegenerative diseases has been reported, including PD patients. An increased HO-1 signal has been detected in reactive astrocytes and affected dopaminergic neurons showing Lewy bodies[49]. Although, the authors suggested that the HO-1 induction was uniquely due to its antioxidant properties, it is clear now that the elevation of HO-1 levels also represents an attempt to downregulate inflammation[50].

A direct proof of the protective effect of HO-1 induction has been obtained in experimental models. The injection of adenovirus containing human heme oxygenase-1 gene (*Hmox1*) into the substantia nigra of

methyl-4-phenylpyridinium (MPP+) treated rats (a model for PD) revealed that the overexpression of *Hmox1* protects dopaminergic neurons by reducing the expression of TNF-α and IL1β, and increasing brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF) expression[51]. Both growth factors, BDNF and GDNF, are able to prevent injury to dopaminergic neurons and improve the behavioural deficits in PD[52-54]. A recent work based on organotypic cultures of substantia nigra (challenged with rotenone to induce PD) demonstrated that inflammation and redox imbalance are early and simultaneous triggers for dopaminergic neuron loss[55]. In this study, *Hmox1* upregulation occurs only at the very first phases of neurodegeneration (3 hours after rotenone challenging, a time which represents the pre-diagnosis stage in human PD based on dopaminergic neuron loss). Similarly, *Bdnf* expression initially increased but then rapidly declined below the control level accompanying dopaminergic neuron demise. The modulation trend of both markers (*Hmox1* and *Bdnf*) was interpreted as a failed tentative reaction to the insult[55], supporting the hypothesis of the interplay between L-DOPA and bilirubin (see above). This interpretation may also agree with a recent finding of Song *et al.*[56], who reported that the expression levels of HO-1 in the saliva of PD patients with early-stage (stage 1) based on the Hoehn and Yahr scale were significantly higher compared to control subjects or PD patients at stage 3 (advanced PD). The authors proposed an additional value of HO-1 as a potential biomarker in idiopathic PD patients. Notably, hyperactivation of HO-1 may exacerbate oxidative stress by the deposition of iron (Fe), one of its products [Figure 2], a phenomena frequently observed in neurological lesions and known to worsen the disease[49]. This aspect is discussed in detail later on in the review.

Contrary to PD and schizophrenia, other neurological conditions have been associated with a reduced TSB level.

Multiple sclerosis

MS is a chronic inflammatory disease characterized by the destruction of myelin in the brain and spinal cord likely due to loss of immune system tolerance to myelin[57]. TSB levels have been reported to be lower (*vs.* healthy controls) also in MS subjects with clinically isolated syndrome (predominately by neuroinflammation) and relapsing-remitting multiple sclerosis (predominately by neurodegenerative disorder), where predominate neurodegenerative disorders[58]. Interestingly, bilirubin levels have been shown to be significantly lower in relapsing-remitting multiple sclerosis compared to clinically isolated syndrome patients[59], suggesting a relationship between increased disease severity and decreased TSB. This hypothesis seems to be confirmed by the study of Ljubisavljevic *et al.*[59] who found a significant correlation between higher TSB and lower disability status, fewer MRI lesions, and shorter disease duration in both study groups. In the brain tissue from MS patients, enhanced nitrotyrosine staining, one of the markers of oxidative damage[60], was found in demyelinated regions, specifically in hypertrophic astrocytes and foamy macrophages on inflammatory lesions[61]. The damaged sites also showed an upregulation of antioxidant enzymes, including HO-1, compared to normal-appearing white matter and white matter tissue from control brains with no neurological disease, and HO-1 immunoreactivity was particularly confined to microglia[61]. These findings emphasize the close interaction between oxidative stress and inflammation in MS.

The effects of bilirubin on MS have also been studied in the animal model for the disease, the so-called experimental autoimmune encephalomyelitis (EAE) model, where the infiltration of lymphocytes, the activation of $CD4^+$ T cells, and death of oligodendrocytes lead to the destruction of myelin sheaths[62,63]. Figure 3 illustrates the most relevant mechanisms of action of bilirubin in EAE, one of the best-known models unravelling the interplay between MS and bilirubin. Bilirubin administration in the EAE model effectively prevented both acute and chronic EAE, even better than did glucocorticoid treatment, the most commonly used therapy for MS[64,65].

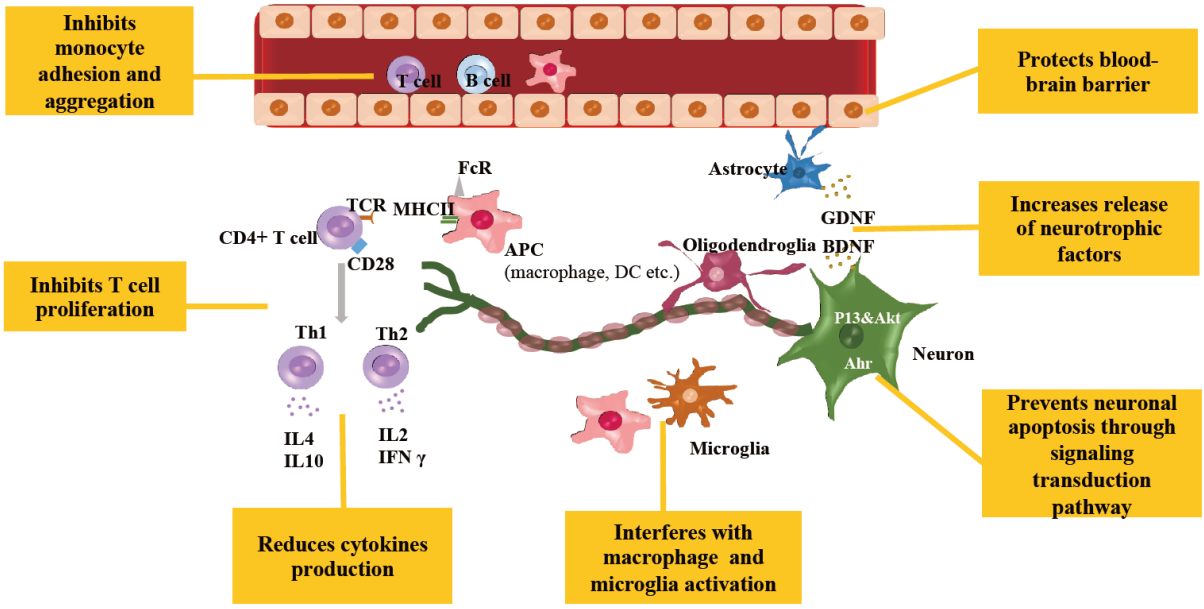

Figure 3. Multiple ways of bilirubin as immunomodulator in neuroinflammation disease. Ahr: aryl hydrocarbron receptor; APC: antigen presenting cell; BDNF: brain-derived neurotrophic factor; DC: dendritic cell; FcR: Fc receptor; GDNF: glial cell line-derived neurotrophic factor; IFNγ: Interferon γ; IL: interleukin; MHC II: major histocompatibility complex; PI3K/Akt: phosphatidylinositol 3-kinase; Th: T helper; TCR: T cell receptor

Histological analyses demonstrated that bilirubin interfered with the infiltration of inflammatory cells into the CNS by protecting the blood-brain barrier from free radical-induced permeability changes[64], stressing the intimal connection between redox stress and inflammation in CNS diseases. Further study supported the antiinflammatory potential of bilirubin in the EAE model[64]. *In vitro* experiments using spleen-harvested $CD4^+$ T cells showed that bilirubin at non-apoptotic concentrations (20-150 μM) inhibits $CD4^+$ T cell proliferation through various mechanisms. It suppresses the production of T helper-1 (Th1) cytokines (IL2 and IFNγ) and Th2 cytokines (IL4 and IL10), reduces costimulatory molecule activity (CD28 on $CD4^+$ T cell, the co-receptor B7-1 activity in macrophages and dendritic cells), inhibits NFkB activation, which is a key transcription factor involved in T cell receptor-mediated signaling, and downregulates inducible MHC (major histocompatibility complex) class II expression. Bilirubin effectively downregulates EAE in SJL/J mice as confirmed by the reduction of the proliferation capacity of $CD4^+$ T cell. Meanwhile, the reduction of endogenous bilirubin synthesis by zinc-protoporphyrin, a specific inhibitor of the bilirubin producing-enzyme HO-1, dramatically exacerbates this disease[66]. In contrast, induction of HO-1 by cobalt protoporphyrin IX (CoPPIX) inhibits EAE effectively[67].

According to the previous data, it seems beneficial to increase bilirubin synthesis through HO-1 induction. Several clinically used drugs have been reported to induce HO-1, among them are nonsteroidal antiinflammatory drugs (e.g., coxibs, acetylsalicylic acid) and hypolipidemic agents (e.g., niacin, fibrates, statins)[68]. Atorvastatin and rosuvastatin treatment in mice demonstrated protection by increasing not only plasma bilirubin concentrations (up to 70%) but also cardiac tissue bilirubin content (up to 119%)[69]. On the contrary, HO-1 induction in astroglia promotes oxidative mitochondrial membrane damage, iron sequestration, and mitophagy (macroautophagy)[56]. These reasons then increase the doubt of bilirubin synthesis through HO-1 induction as a good strategy to counteract the neuroinflammatory process.

In another part of inflammation signalling pathways, both UCB and biliverdin are known as activators of aryl hydrocarbon receptor (AhR), a ligand-activated transcription factor that plays critical modulatory roles in various immune cells during innate and adaptive immune responses[2,70,71]. AhR has critical roles

in MS by modulating IL17 producing T-helper (Th17) cells, and regulatory T cells[72-74], as well as B cells, macrophages and dendritic cells. In macrophages, AhR regulates IL1β production and also IL6, IL12 and TNFα expression[73,75]. AhR also inhibits the transcriptional activity of NFκB in stimulated macrophages[76]. Meanwhile, in dendritic cells, AhR mediates the generation of T regulatory cells and Th17 cells from naive T cells and also IL10 production[77]. Notably, the injection of low doses of UCB (20 μg/kg body weight, injected into C57Bl/6 mice) increased the population of regulatory T cells by more than 50% compared to controls, prolonging graft survival[78]. Regulatory T cells are also inducible by HO-1 activity, CO, and UCB[78] and all "yellow players" [Figure 2].

Optic neuritis and neuromyelitis optica

Reduced TSB has been noticed in both optic neuritis, an acute inflammatory demyelinating disorder of the optic nerve, and neuromyelitis optica, also known as Devic disease, a severe autoimmune demyelinating disease that selectively affects the optic nerve and spinal cord[79-82]. Although the main focus was on the potential antioxidant role of bilirubin, suggesting that the low TSB is a result of the overconsumption of bilirubin due to oxidative stress, immunohistochemistry of damaged nerves is similar as in MS[83], with an evident inflammatory component. Thus, the interplay between bilirubin and inflammation described in MS also applies to optic neuritis and neuromyelitis optica. Further studies are needed.

Diabetic retinopathy and stroke

TSB levels have been found to be decreased also in patients with diabetes mellitus and diabetic retinopathy *vs.* subjects with diabetes mellitus without retinopathy, suggesting TSB as a biomarker of diabetic retinopathy[84]. In the pathogenesis of diabetic retinopathy, particularly in the non-proliferative type, hyperglycemia exposure causes the alteration of retinal microvasculature with pericyte loss[85], endothelial apoptosis and endothelial thickening of the basement membrane, which further lead to capillary occlusion and ischemia[86].

Similarly, damage and even destruction of the cerebral vasculature are landmarks of stroke and related vascular events[87]. In a large study conducted by Li *et al.*[88], 343 subjects with silent cerebral infarct presented with lower TSB levels. In another study, patients with deep white matter lesions (DWMLs) had a decreased TSB level compared to non-DWMLs subjects. Again, the low and intermediate bilirubin groups showed a higher prevalence of severe DWMLs than did the group with higher bilirubin levels[89]. Notably, DWMLs are a recognized predictor for the development of impaired cognitive function and stroke[90,91].

A recent study also explored the association of UCB and intracranial atherosclerosis, which was found in approximately 50% of patients with transient ischemic attack and up to 47% of ischemic stroke patients in Asia[92-94]. In this study, UCB was significantly negatively associated with intracranial atherosclerosis. The odds of intracranial atherosclerosis was 0 0.67-fold lower in participants in the high UCB concentration group (≥ 10.10 mMol/L) when compared with those in the low UCB concentration group[94]. An increased bilirubin level has been proposed as a novel independent predictor for hemorrhagic transformation and symptomatic intracranial hemorrhage after mechanical thrombectomy[95].

The role of bilirubin in all previously mentioned conditions might be explained by a large number of studies suggesting bilirubin as a protector against microvascular complications. Bilirubin exhibits potent antiinflammatory effects [via HO-1, nuclear factor erythroid 2-related factor 2 (Nrf2) and NFκB] by the inhibition of monocyte transmigration [through a decrease in TNFα-induced monocyte chemoattractant protein-1 (MCP-1) secretion], reducing endothelial vascular cell adhesion molecule-1 (VCAM-1) expression, and improving endothelial cell dysfunction and hyperproliferation after damage[96-99]. UCB also appears to affect the immune system by inhibiting the activation of the complement cascade[100], and also by modulating the phagocytic and antigen-presenting function of macrophages (by changing the expression of Fc receptors)[101].

The potential protective role of HO-1 induction has been demonstrated in several studies. HO-1 increases early (24 h) after brain trauma[102], and in intracerebral haemorrhage models, after infiltration of the brain by blood components activating the inflammatory response[103]. HO-1 activity is observed in both endothelial cells and microglia surrounding the haematoma site. The protective effects of HO-1 in neurons was studied by Orozco-Ibarra *et al.*[104], who revealed that the upregulation of HO-1 prevented the death of cerebellar granule neurons due to mitochondrial toxicity. Further investigation found that the HO-1 products, bilirubin and CORM2 (carbon monoxide releasing molecule), were involved in preventing cell death.

HO-1 has neuroprotective effects by regulating the phosphatidylinositol 3-kinase (PI3K/AKT) signalling pathway and by reducing apoptosis in rats with cerebral haemorrhage[105]. Feng *et al.*[106] showed that the PI3K/AKT and extracellular signal regulated kinase pathways are involved in oleanolic acid induced HO-1 expression by activating Nrf2 in vascular smooth muscle cells. As one of the signalling pathways for cell survival, the PI3K/AKT signal transduction pathway plays an important role in cell proliferation and differentiation and inhibition of neuronal apoptosis[107]. Therefore, HO-1 may protect the nerves of rats with cerebral haemorrhage by regulating the PI3K/AKT signalling pathway.

A second isoform of HO exists in the brain, called heme oxygenase-2 (HO-2). Considered constitutive, it looks like HO-2 in the brain plays the vital function of maintaining adequate levels of UCB to guarantee normal cellular homeostasis, participating in brain protection[108]. The protective activity of HO-2 has been reported in intracerebral haemorrhage models, where heme oxygenase-2 gene (*Hmox2*) deletion led to greater brain injury volumes and neurological deficits than in wild-type mice after intracerebral haemorrhage[109]. In brain cultures, Doré *et al.*[108] expanded our knowledge on the role of HO-2 and bilirubin as neuroprotective factors by showing increased neuronal death in cerebellar granule cultures of *Hmox2* knockout mice (*Hmox2*-/-). On the contrary, *Hmox2* transfection in human embryonic kidney 293 (HEK293) cells rescued cells from apoptotic death. In another study, the induction of HO-2 activity by phorbol esters enhanced the production of bilirubin, which protected primary hippocampal and cortical neuronal cultures from the neurotoxicity of H_2O_2. Of note, HO-2 was immunolocalised in neurons both before and after traumatic brain injury, whereas HO-1 was highly upregulated in glia only after traumatic brain injury. Cell loss was significantly greater in *Hmox2*-/- mice in areas including the cortex, hippocampus and lateral dorsal thalamus[110].

Biliverdin (BV), another yellow player, also showed neuroprotective effects by ameliorating cerebral reperfusion injury in rats most probably via its antiinflammatory activity[111]. Two pathways are known to be involved in the antiinflammatory mechanism of BV: by activating the nitric oxide-dependent biliverdin reductase, BV reduces the expression of toll-like receptor-4 (TLR-4) in murine macrophages[112], and BV regulates the expression of complement C5a receptor[113]. Furthermore, a study by Zou *et al.*[111] found the downregulation of miR-204-5p and its target gene, ETS protooncogene 1 (Ets1), in cerebral ischemia-reperfusion injury rats following BV administration. Ets1 is known to be responsible for inducing proinflammatory Th1 type response and causing neuronal death[114]. Therefore, BV may play a crucial role in preventing injury in stroke by interfering in miRNAs levels.

Additional neurological conditions underpinned by reduced TSB levels

ALS

In ALS, the reduction of TSB levels correlates with both the clinical state and disease duration. Patients with long-lasting ALS (where motor neuron degeneration is noticed) have lower TSB levels than do patients with a shorter duration[115].

Seasonal affective disorder

Nocturnal plasma bilirubin levels were evaluated in individuals with unipolar recurrent winter seasonal major depressive disorder and age-/gender-matched controls. Lower bilirubin levels were found in patients with seasonal affective disorder. The reduction in bilirubin level in this study was proposed as a vulnerable risk for depression, although only an associative and not a causative link was provided[116].

Dementia

A significant reduction in TSB levels was reported in patients with cognitive impairment compared to those with normal cognitive function, although no significant correlation between bilirubin and disease duration was found[117]. In a further study by Baierle *et al.*[118], the authors showed that the significant increase in the proinflammatory cytokines IL1β and TNFα correlated with the increase in oxidative stress and inversely associated with low cognitive performance in elderly patients.

AD

As in the majority of neurological conditions, TSB levels are also decreased in AD[119]. The lower concentration of bilirubin is supposed to have a role in the transition from mild cognitive impairment to clear AD[120]. Similarly, reduced *Hmox1* mRNA[121] and protein[122] levels were detected in the serum of full-blown AD subjects, and proposed to correlate with cognitive decline[120]. Notably, the diagnosis of AD and cognitive decline is usually a late event and performed after the pathological alteration has already existed for some time. In the early stages, the brain tries to protect itself from the oxidative and inflammatory insult of AD by increasing the endogenous production of UCB. Both HO-1 and biliverdin reductase A (BLVR-A), the two key enzymes in biliverdin and bilirubin production [Figure 2], have been reported to be upregulated in the brain of AD patients[103,123]. However, the upregulation of HO-1 will also cause Fe deposition in the brain, which worsens and accelerates disease progression by enhancing the redox imbalance in ongoing AD lesions[49,124-126]. It should be recalled that the brain possesses very low antioxidant capacity compared to other organs, being much lower in neonatal life[127,128] and in the elderly[129,130]. Thus, the enhanced pro-oxidant milieu due to HO-1 hyperactivation leads to an increased oxidative and nitrosative post-translational modification of cellular enzymes, with their consequent inactivation[131]. One of the targets of this mechanism is BLVRA, and thus, UCB production will be stopped[123].

The chains of events described here, leading to the disruption of UCB-mediated protection and contributing to the progression of neurological damage fits well with what we described in PD.

IS THE BRAIN DIFFERENT FROM THE REST OF THE BODY?

Differently from extra-CNS diseases involving oxidative imbalance and inflammation, where a positive correlation between higher TSB level (Gilbert syndrome-like) and reduced disease incidence has been frequently found[2,3,68], the most readily evident finding in this review is that brain diseases have lower TSB levels in common. Two major explanation have been given: (1) lower TSB levels in subjects with neurological disease may reflect the consumption of UCB due to oxidative stress, although it seems unlikely that the local redox imbalance occurring in the brain may affect the systemic levels of bilirubin; and (2) individuals with lower TSB, thus a lower systemic antioxidant status, might be more vulnerable to oxidative stress[118] and related diseases (both in the brain and extra-CNS organs) [Figure 1].

Experimental evidence strongly supports the notion that an increased concentration of bilirubin in the CNS exerts antiinflammatory and antioxidant effects. On the other hand, it should be recalled that hyperactivation of HO-1 in the CNS, reported to be a tentative reaction against brain insults, may enhance the damage[68,104,131-148]. This specificity of the CNS has to be taken into account in the growing research aimed at modulating the bilirubin players to increase levels of bilirubin and the protection it confers.

CONCLUSION

Studies assessing clearly the molecular events correlating bilirubin and neurological damage are still lacking, and many conclusions are based on clinical series. Understanding the role of bilirubin and all the other yellow players in the development and progression of the different neurological disorders may help in deciphering the efficacy of the modulation of bilirubin level to prevent CNS diseases. However, due to the side effects of the drugs inducing HO-1 activity, caution must be taken before their recommendation. More intriguing and promising is the *in situ* CNS elevation of UCB level achievable by the development of molecules modulating BLVR. The future will hopefully provide the much-needed answer due to the medical and social burden of neurological disorders.

DECLARATIONS

Authors' contributions

Made substantial contributions to the search of the literature, the writing of the manuscript, and the revision and discussion of the contents of the paper: Jayanti S, Moretti R, Tiribelli C, Gazzin S

Availability of data and materials

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Conflicts of interest

All authors declare that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Review

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Cerebrospinal fluid biomarkers in idiopathic normal pressure hydrocephalus

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Abstract

Idiopathic normal pressure hydrocephalus (iNPH) is characterized by abnormal cerebrospinal fluid (CSF) flow and consequent cerebral ventricular enlargement due to imbalance of CSF production and absorption. The typical triad symptoms, namely cognitive decline, gait disturbance, and urinary incontinence, are thought to be caused by disruption of CSF circulation. However, some patients may still experience symptomatic progression after functional shunting, suggesting that iNPH is far more complicated than a simple disorder of CSF circulation. Moreover, the diagnostic workup of iNPH can be challenging due to symptomatic and neuroimaging overlaps with other neurological disorders, such as Alzheimer's disease. Furthermore, accumulating studies indicate that the pathogenesis of iNPH might relate to multiple mechanisms, including abnormalities of brain development, brain extracellular matrix, synaptic function, blood flow, and cerebral metabolism. Therefore, iNPH is not an isolated entity in occurrence and development. Nevertheless, different pathogeneses may result in protein content changes in CSF, and the biomarkers in CSF may reflect the possible mechanisms involving the etiology of iNPH and are potentially useful in assisting the diagnosis and treatment selection. In this review, we summarize the main findings of CSF biomarkers and aim to outline a possible synthetic profile in assisting iNPH diagnosis and therapeutic options.

Keywords: Idiopathic normal pressure hydrocephalus, cerebrospinal fluid, biomarkers, neurodegenerative diseases

INTRODUCTION

Idiopathic normal pressure hydrocephalus (iNPH) is one of the disabling neurological disorders whose potential treatability is significantly impacted by the timeliness of unequivocal diagnosis. iNPH is characterized by ventriculomegaly that is caused by an imbalance between cerebrospinal fluid (CSF) production and absorption. The characteristic triad symptoms of dementia, gait disturbance, and urinary incontinence are thought to be caused by a disruption of CSF dynamics[1,2]. Therefore, the triad symptoms of iNPH could be surgically treatable with a diversion of CSF into peritoneal cavity or heart[3]. However, the diagnostic workup of iNPH can be a challenge due to neuroimaging and symptomatic overlaps with other neurological disorders, such as Alzheimer's disease (AD) and subcortical ischemic vascular disease, especially at early stage. Moreover, although the cognitive decline could be proceeded by these diseases, they are not equally responsive to the treatment of CSF shunting. Therefore, further effort to improve the diagnosis of iNPH would benefit the current imaging and symptomatic diagnostic criteria. Increasing studies indicate that the pathogenesis of iNPH involves multiple mechanisms, including abnormalities of brain development, brain extracellular matrix, synaptic function, blood flow, and cerebral metabolism, which could result in protein content changes in CSF. On the other hand, impaired CSF absorption could lead to a pathological flow of CSF into the periventricular tissues to initiate a cascade of pathological processes such as edema and consequent neuronal degenerative changes[4]. Therefore, measurements of different biomarkers in CSF may reflect the underlying neuropathological changes of the brain and could play an important role in revealing the possible etiological mechanisms. Furthermore, its detection may facilitate the timeliness and accuracy of iNPH diagnosis, and thus becomes potentially useful for therapeutic selection and treatment response monitoring. In addition, the biomarkers could help to differentiate iNPH from other neurological disorders, which might mimic iNPH symptomatology but show unsatisfactory outcomes after shunting[5,6]. Despite a growing interest, the CSF biomarker profile in iNPH has not yet been identified definitively. In this review, we summarize the main findings of CSF biomarkers regarding iNPH and outline a rough CSF profile in order to assist iNPH diagnosis and provide adequate treatment. It is notable that, due to the etiological complexity of iNPH, most biomarkers might lack specificity for iNPH diagnosis and are possibly coincidental, confounding with other overlapping neurological diseases. In addition, in comparison with a cortical brain biopsy or neuropsychological testing, biomarkers may also have limitations in distinguishing iNPH from comorbid iNPH plus AD[7], as well as in predicting clinical cognitive outcome post shunting[6,8,9]. However, a combination of more than one biomarker may enhance the predictive value and provide more viable and accurate solutions. Ideally, the dynamic changes of biomarker measured before and after surgical diversion of CSF would supply useful clinical information for the diagnosis and assistance in monitoring disease progression. The biomarkers could be categorized as AD discrimination, neurodegeneration and demyelination, neuroinflammation, neuropeptides and cerebral metabolites, and as biomarkers in response to cerebral and vascular insulting, among others[1,2,10,11].

BIOMARKERS FOR AD DISCRIMINATION

Dementia in iNPH is potentially reversible if adequately treated. However, it often resembles the clinical appearance of patients with AD, such as memory decline, as well as attention and executive impairment[12]. Urinary incontinence and gait disturbance may also occur in both diseases due to disturbed subcortical network caused by vascular pathology. Moreover, ventricular enlargement may have been observed in AD patients as a result of cerebral atrophy rather than CSF circulation impairment[13]. Furthermore, the pathological examination of cortical brain biopsies performed during placement of CSF shunts revealed AD neurodegenerative changes in 24% of iNPH patients, suggesting a high comorbidity of both diseases. Thus, cortical brain biopsy may provide a valuable predictive way for outcome evaluation[6,8]. However, cortical brain biopsy is not always available or appropriate in some cases. Moreover, both iNPH and AD diseases may manifest sleep disturbances, which correlate with dysfunction of the glia-lymphatic (glymphatic)

system, consequently building-up of brain metabolic wasters, favoring dementia development[14,15]. Therefore, it is always a challenge to discriminate iNPH and AD diseases in clinical practice.

The glymphatic system facilitates cerebral metabolite and brain fluid clearance during sleep via glia-supported perivascular channels. This system facilitates efflux of cerebrospinal and interstitial fluid via the perivascular spaces to the meningeal and cervical lymphatic vessels, assisting the draining/clearing of metabolic wastes from the central nervous. The glymphatic flux is proposed to be driven by cardiac-induced arterial pulsation[16], and may be possibly manipulated through change of intracranial pressure pulsatility with our cardiac-gated device[17]. Most interestingly, the action of glymphatic flux is predominant during sleep[18], and up to 90% of iNPH patients are associated with obstructive sleep apnea, a common sleep disorder[19]. Blockage of the airway in obstructive sleep apnea causes increased awakenings and decreased quality of sleep, resulting in glymphatic dysfunction and increased cerebral Aβ aggregation[20]. Patients with obstructive sleep apnea encounter reduced oxygen intake due to intermittent airway obstruction. Excessive breathing against a closed airway induces negative intrathoracic pressure, sufficient to cause atrial distortion and reduced venous return to the heart[19] and ultimately affect arterial pulsation, resulting in dysfunction of glymphatic flux.

Many studies have shown impaired glymphatic function in both iNPH and AD. Furthermore, iNPH and AD patients share multiple clinical and pathologic features such as Aβ deposition, cerebrovascular inflammation, impaired localization of perivascular astrocyte aquaporin-4 (AQP4), and sleep disturbances[15]. Therefore, it is a diagnostic challenge in daily practice for iNPH and AD. Although many biomarkers have been investigated for their discrimination, amyloid-β 42 ($A\beta_{42}$), total-tau (t-tau), and phosphorylated tau (p-tau) are the most robust candidate markers to discriminate iNPH from AD patients[1,2]. $A\beta_{42}$ is lower in both iNPH and AD patients compared with healthy control, and $A\beta_{42}$ does not separate iNPH and AD. Tau protein is a microtubule-associated protein and is a marker for neuronal degeneration[21]. The levels of t-tau and p-tau are higher in AD patients compared with iNPH patients and controls, whereas the levels of t-tau and p-tau are within normal range in iNPH patients. The combination of these biomarkers, i.e., the reduced $A\beta_{42}$ with concomitant normal or reduced t-tau and p-tau levels in iNPH coupled with reduced $A\beta_{42}$ with concomitant increased both t-tau and p-tau levels in AD, may significantly improve the accuracy of differential diagnosis between AD and iNPH patients[22]. The mechanism of lower $A\beta_{42}$ level in iNPH patients is unknown. However, the reduced production of $A\beta_{42}$ due to a decline in brain metabolism in the periventricular zone in iNPH patients[23,24] and interstitial Aβ deposition due to impaired glymphatic function may be possible reasons[15]. Meanwhile, the low concentrations of CSF t-tau and p-tau do not support the major cortical degenerative process in iNPH[24,25], whereas, in AD patients, the core pathological changes are the accumulation of abnormally folded beta-amyloid and tau proteins in the plaques and neuronal tangles[26], and the progressive deposition of amyloid plaques lowers $A\beta_{42}$ level. Moreover, concurrent axonal degenerations and neurofibrillary tangle formation further increase t-tau and p-tau CSF levels in AD patients[27]. The representative information and main biomarkers for assisting differential diagnosis of iNPH and AD are summarized in the attached Table 1.

NEURODEGENERATION AND DEMYELINATION

The disturbance of CSF circulation could lead to a potentially hostile milieu for cerebral structures, especially periventricular areas and subcortical structures, and could result in vascular lesions, destruction of periventricular white matter, and subsequent neurodegeneration and demyelination[28-30]. Such pathological changes could be estimated with the examination of CSF contents, such as neurofilament light chains (NFL), myelin basic protein (MBP), and leucine-rich-α2-glycoprotein (LRG)[31-33]. NFL is a cytoskeletal element in nerve axons and dendrites, and therefore could be considered as a biomarker for axonal damage in patients with iNPH[31,34]. Although some studies did not find difference of CSF NFL

Table 1. The representative information and biomarkers in iNPH and AD

	iNPH	AD	Referencs	Comments
Etiology	Multiple	Multiple	[1-4]	
Dementia	10%	60%-70%	[12,15]	
Ventriculomegaly	↑↑	↑	[6,13]	18-42% of iNPH also had AD brain biopsy findings
OSA	65%-90%	44%	[15,19]	OSA: obstructive sleep apnea
GFD	Yes	Yes	[14-15,18]	GFD: Glymphatic flux dysfunction
WMLs	↑	↑	[64-66]	WMLs: cerebral white matter lesions
PWMD	↑	↑	[28-30]	PWMD: periventricular white matter damage
FSO	↑↑	↑	[1-3]	FSO: favorable surgical outcome
*Aβ42	↓	↓	[1-2,22-24]	Amyloid-beta-42. No difference *vs.* AD, ↓ *vs.* control
*t-tau	↓/-	↑	[1-2,21-24,27]	Total tau. ↓ *vs.* AD, no difference *vs.* control
*p-tau	↓/-	↑	[1-2,22,27]	Phosphorylated tau. ↓ *vs.* AD, no difference *vs.* control
NFL	↑	N/A	[12,31-34]	Neurofilament light chains. Correlated with PWMD and FSO
MBP	↑	↑	[31-33,39-41]	Myelin basic protein. Correlated with PWMD and FSO
LRG	↑	↑	[31-33,43]	Leucine-rich-α2-glycoprotein
TNF-α	↑	N/A	[45-46]	Tumor-necrosis factor α. Correlated with FSO
TGF-β1	↑	N/A	[47-49]	Transforming growth factor β1
IL-1β	↑	↑	[44,50-52]	Pro-inflammatory cytokines, interleukin-1β
IL-6	↑	↑	[50-52]	Pro-inflammatory cytokines, interleukin-6
IL-10	↑	↑	[50-52]	Anti-inflammatory cytokine, interleukin-10
TFPI-2	↑	↑	[50-52]	Tissue factor pathway inhibitor 2
YKL-40	↑	↑	[50-53]	Chitinase-3-like protein-1
MCP-1	↑	↑	[50-52]	Monocyte chemoattractant protein-1
SOM	↑/↓	N/A	[10,54-55]	Somatostatin
VIP	↓	N/A	[10,54-55,57]	Vasoactive intestinal peptide
NPY	↓	N/A	[10,54-55]	Neuropeptide Y
DSIP	↓	N/A	[10,54-55]	Delta-sleep inducing peptide
NGF	↑↑	N/A	[69,70]	Nerve growth factor
VEGF	↑	N/A	[59,71-73]	Vascular endothelial growth factor. Correlated with FSO
GFAP	↑	N/A	[34,76]	Glial fibrillary acidic protein
PGDS	↓	-	[77]	Prostaglandin D synthase

*Strengths; other weaknesses. ↑: increased; ↓: decreased; -: normal; N/A: not avaiable; iNPH: Idiopathic normal pressure hydrocephalus; AD: Alzheimer's disease

levels between iNPH and AD patients[11,32], as well as controls[35], other studies demonstrated increased CSF NFL levels, and the increase paralleled the degeneration of large myelinated axons in iNPH[31,36]. In addition, some studies observed that the ventricular NFL level directly correlated with altered signals in periventricular white matter in brain MRI[37]. Moreover, one study demonstrated that high preoperative NFL level was associated with favorable surgical outcomes, and suggested that NFL could possibly be used as an indicator for neurodegeneration and a marker of ongoing axonal damage[38].

Demyelination of the periventricular white matter could occur in hydrocephalus due to the result of mechanical stretching. MBP is an oligodendroglial structural protein of myelin and sulfatide is a glycosphingolipid component of myelin, and they are essential for the maintenance of central nervous system myelin and axon structure[32,39]. Both MBP and sulfatide are well known indicators for ongoing demyelination and therefore are attractive markers for the pathological process[40]. However, the CSF levels of MBP are higher in many different neurologic disorders, including iNPH and cerebrovascular diseases, leading to lack of specification for iNPH diagnosis[32,36], whereas it is demonstrated that changes of MBP levels are correlated with periventricular white matter damage[41]. When comparing the levels of MBP pre- and post-shunting, the results showed that the levels of MBP decreased post-shunting, suggesting that MBP could be used for evaluation of brain damage and shunting effect[42].

LRG is an astrocytic protein and could be induced by inflammation. The LRG level in CSF increases with age in iNPH and other dementia diseases. It was speculated that the accumulation of LRG in the brains is

one of the causes of neurodegeneration, therefore its level in CSF could be an anticipated marker for early diagnosis of iNPH and other dementia diseases[33,43].

Taking together, all these markers allow tracking the integrity of periventricular and subcortical structures. Although they are not disease specific, their changes in CSF directly reflect cerebral damage, and they may be useful indicators in comparative analyses between iNPH and other neurodegenerative diseases.

NEUROINFLAMMATION

Cytokines mediate inflammatory response and often correlate with neurodegeneration in neurological diseases. The profile of CSF cytokines provides access to explore the pathogenic mechanisms of different neurological diseases and therapeutic approaches[44]. Abundant CSF cytokines have been investigated in iNPH patients, but a more definite profile still needs to be clarified[32,36].

Tumor-necrosis factor (TNF-α) is a cytokine of inflammatory mediator and its level in CSF is significantly high in iNPH patients[45,46]. Most interestingly, the CSF level of TNF-α returned to the control level in the patients with shunt improvement. Because of its short half-life, the increased CSF TNF-α may be caused by increased production rather than the accumulation due to CSF stagnation, which suggests that TNF-α in CSF might be used as a candidate marker for the evaluation of demyelination and disease progression in iNPH patients. More studies are needed for validation.

Transforming growth factor β1(TGF-β1), one of the three cytokines in the TGF family, plays a role in cell differentiation and tissue modification during brain development. It could be released from microglia and astrocytes in response to cerebral insult to initiate neuroinflammation and neurodegeneration through the induction of fibrosis, vascular hypertrophy, accumulation of extracellular matrix components, and neuronal apoptosis[47-49]. TGF-β level was found to be higher in iNPH patients than controls, and was considered to be a reliable index of cerebral damage in iNPH[49].

Other increased inflammatory biomarkers measured in iNPH patients include IL-1β and IL-6 (pro-inflammatory cytokines), IL-10 (anti-inflammatory cytokine), tissue factor pathway inhibitor 2 (TFPI-2), chitinase-3-like protein-1 (YKL-40), and monocyte chemoattractant protein (MCP-1)[50-52]. However, as similar changes are also observed in AD and Parkinson's disease, these changes only reflect an underlying neuroinflammatory processes of pro-inflammatory reaction (IL-1β and IL-6) and compensatory anti-inflammatory reaction (IL-10), rather than disease-specific indicators[44,51,52]. TFPI-2 is involved in inflammatory process by recruiting astrocytes and microglia to the injury site[50]. YKL-40 is then released from astrocyte and/or microglia in response to neuroinflammation. The increased CSF YKL-40 levels seem to be correlated with cognitive decline and therefore to predict progression of dementia[53]. However, more studies are deserved on the clinical use of this novel promising neuroinflammation biomarker[35,48].

NEUROPEPTIDES AND CEREBRAL METABOLITES

Neuropeptides, including somatostatin, vasoactive intestinal peptide, neuropeptide Y, and delta-sleep inducing peptide, have been evaluated by various groups[10,54,55]. Decreased CSF somatostatin levels suggest damage to the hypothalamus and the cortical neurons that normally have high concentrations of somatostatin[54]. Higher level of somatostatin correlates with better visual memory and mental condition in iNPH patients, proposing that somatostatin may have a modulatory role in cognition[10]. Vasoactive intestinal peptide is a potent vasodilator and therefore may play a role in chronic ischemia, and the CSF level is usually higher in iNPH patients with cerebrovascular disease[55-57]. Delta-sleep inducing peptide is a nine-amino acid peptide with a role in sleep-wakefulness regulation. iNPH patients with lower delta-sleep inducing peptide level show worse psychomotor performance[56]. Several studies also reported reduced levels of neuropeptide Y in iNPH patients[54-56].

Cerebral metabolism changes may occur in iNPH patients. iNPH patients were also reported to have altered levels of lactate, an end product of anaerobic glycolysis underlying a presence of chronic ischemia[58,59]. Free-radical peroxidation could result in cellular dysfunction and may therefore be implicated in the pathogenesis of iNPH and dementia. A study showed that the levels of free-radical peroxidation products significantly increased in iNPH patients[60]. The authors implied that peroxidation of cytoplasmic membranes might be involved in the development of cognitive dysfunction in iNPH.

BLOOD-BRAIN BARRIER CHANGE AND BIOMARKERS RESPONDING TO CEREBRAL AND VASCULAR INSULTING IN INPH

Blood-brain barrier is a physically powerful gateway that strictly monitors and controls the interchange of substances between central nervous system and blood flow[61]. Its function is strictly dependent on the integrity of microvascular endothelium and thus affected by many pathophysiological risk factors, including vascular/hemodynamic changes, inflammation, *etc*., and in turn affects the homeostasis of central nervous system[62]. The "CSF/blood albumin ratio" represents a reliable index of blood-brain barrier function. Blood-brain barrier impairment was reported in different neurodegenerative diseases, including AD and cerebral vascular disease[63]. Nowadays, it has been scarcely evaluated in iNPH patients, but available reports indicate a substantial preservation of the blood-brain barrier[22,36].

Vascular risk factor may be a component of subcortical neuropathology in the development of iNPH[2]. As key components, cerebral white matter lesions and hypertension were reported to be related to the pathophysiology of iNPH[64-66]. White matter lesions, involved in different cognitive processes and/or clinical outcomes, are associated with small vessel disease and white matter ischemia. The association between iNPH and white matter lesions indicates the involvement of microvascular disturbances in the white matter and in the pathological processes of iNPH. In addition, hypertension increases the risk of iNPH through the mechanisms of involved small vessel diseases, including hypertension induced endothelial damage and resultant extravasation of blood products into white matter, impaired blood flow with reduced metabolism, and direct mechanical effect on ventricular size[66,67]. Therefore, identifications of vascular related risk factors may improve diagnostic accuracy and address the underlying pathology regarding the development of iNPH, and ultimately provide suitable intervention for iNPH management. Overall, the dynamic and morphological alterations in subcortical structure of iNPH brain could be resulted from white matter lesions, hypertension related vascular lesions, destruction of periventricular white matter axons and gliosis, and impaired CSF circulation[28]. Such pathological alterations could affect CSF protein contents and biomarkers in CSF could mirror the underlying pathologic alterations. As markers of subcortical damage, at least three proteins have been measured in iNPH patients, including NFL, LRG, and MBP[1]. The functions and clinical application of these proteins are discussed above. In summary, NFL is a cytoskeletal protein for maintenance of axonal architecture and is considered as a marker for neuronal morphological integrity[31]. Although it has also been assessed as a biomarker for inflammatory and neurodegenerative diseases, it has been observed that ventricular NFL levels in iNPH patients directly correlate with more extensive altered signals in periventricular white matter in brain MRI[1]. LRG is an astrocytic protein and is increased in CSF of iNPH patients, suggesting a potential biomarker for iNPH, but it also changes with aging and non-specific inflammation[68]. MBP is an oligodendroglial structural protein of myelin. Its CSF levels are increased in iNPH patients and other cerebrovascular and neurodegenerative diseases, indicating the damage of periventricular white matter[1,32].

In addition, nerve growth factor (NGF) play an important role in neuro-regeneration in response to brain injury and age-related atrophy. NGF is scarcely detectable in innervated tissues, but denervation of cerebral tissue could lead to the production of NGF and become measurable in the target tissues[69]. The CSF level of NGF was found to be significantly higher in hydrocephalus patients compared with the controls[70], which

suggests the possibility that the increased NGF levels could represent an increased cerebral regeneration after shunting.

Vascular endothelial growth factor plays roles in many cerebral physiological and pathological modifications, and its level in CSF is respondent to ischemic condition involved in different neurological disorders[59,71-73]. Our group demonstrated that the CSF levels of vascular endothelial growth factor in iNPH patients have circadian variations and exercise induced increasing[74]. The higher concentration of vascular endothelial growth factor level in CSF is associated with less response to shunting and worse clinical outcome, suggesting a possible concurrent ischemic or vascular injury in iNPH patients[73,75].

Glial fibrillary acidic protein is a marker for gliosis[34,76]. In iNPH patients, the CSF level of glial fibrillary acidic protein was increased when compared with controls, and correlated with disease progression[38]. The increased glial fibrillary acidic protein level in CSF suggests an irreversible damage to astrocytes, since glial fibrillary acidic protein is not secreted by astrocytes.

All of these markers suggest the involvement of vascular risk factors and consequent subcortical white matter lesions in the development of iNPH; however, further studies are needed to explore their predictive value in clinical application.

OTHER BIOMARKERS AND METHODOLOGICAL IMPACT ON CSF BIOMARKER DETECTION

The level of prostaglandin D synthase was found to be significantly lower in iNPH patients compared with controls and other dementia patients, such as Lewy body dementia, vascular dementia, and AD[77]. This enzyme is secreted into CSF by the leptomeninges and the trabecular cells of the arachnoid membrane. The authors speculated that the decreased level of prostaglandin D synthase was probably due to a degenerative change of the arachnoid membrane in iNPH patients.

Finally, the methodology of CSF biomarker detection may also affect the ability to reliably evaluate biological biomarkers for the differentiation and prognosis of cognitive impairment diseases[78]. Many factors may affect the reliability and sensitivity of biomarker detection, for example, the systematic difference between different assays, different pre-analytical protocol for sample preparation and storage, analytical variability of measurement procedures, *etc.*[79,80]. When interpreting measurement results, these factors should be considered. In addition, some biomarkers exhibit periodic concentration patterns. Therefore, the most appropriate time for sample collection must also be considered when designing a protocol[79].

CONCLUSION

The overlap of neuroimaging and symptomatic manifestations leads to diagnostic confusion between iNPH and other neurodegeneration diseases, such as AD and subcortical ischemic vascular disease. Despite the absence of definite pathological hallmarks, the biomarkers altered in CSF might serve as targets for diagnosis and therapeutic intervention. Furthermore, the biomarkers in CSF could reflect the adjacent cerebral pathophysiological status, therefore are potentially useful to provide insight into the pathological changes in the brain milieu and underling pathogenesis. Although many CSF biomarkers have been analyzed in iNPH patients, the significant findings include the reduced $A\beta_{42}$ with concomitant normal or reduced t-tau and p-tau levels in iNPH coupled with reduced $A\beta_{42}$ with concomitant increased both t-tau and p-tau levels in AD. This characteristic alteration may significantly improve the accuracy of differential diagnosis between AD and iNPH patients. Other biomarkers may lack specification in differential diagnosis, but the definite changes may mirror the underlying pathogenesis mechanisms, such as demyelination, neurodegeneration, and neuroinflammation, and provide valuable information to further explore the pathogenesis mechanisms and optical therapeutic manipulations.

DECLARATIONS

Authors' contributions

Conceived of the presented idea: Yang J
Underwent literature review and synthesized a draft: Yang J, Zhang XJ, and Guo J
Contributed ideas throughout the process and approved the final draft: Yang J
Looked over and edited draft: Yang J, Zhang XJ, and Guo J

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Review

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Cerebrospinal fluid amyloid beta and tau proteins in atypical Parkinsonism: a review

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Abstract

Progressive supranuclear palsy, corticobasal degeneration, multiple system atrophy and dementia with Lewy bodies are the most common causes of atypical Parkinsonism and enter the differential diagnosis of Parkinson's disease. multiple system atrophy, dementia with Lewy bodies and Parkinson's disease are synucleinopathies, whereas progressive supranuclear palsy and corticobasal degeneration are tauopathies. Multiple cerebrospinal fluid markers have been applied on cohorts of patients with Parkinsonism, with the aim to develop biomarkers for these disorders. Total tau (τ_T), phosphorylated tau at threonine 181 (τ_P-181) and amyloid-beta with 42 amino acids ($A\beta_{42}$) are considered classical biomarkers for Alzheimer's disease. The aim of the present study is to review the literature regarding these classical cerebrospinal fluid biomarkers in cohorts with Parkinsonism, as well as present data on novel approaches regarding analysis of these proteins.

Keywords: Biomarkers, cerebrospinal fluid, progressive supranuclear palsy, corticobasal degeneration, multiple system atrophy, dementia with Lewy bodies, tau protein, phosphorylated tau protein, amyloid beta

INTRODUCTION

Parkinson's disease (PD) is the most common neurodegenerative movement disorder. The pathologic hallmarks of PD are Lewy bodies, which consist of intraneuronal cytoplasmic depositions of pathological α-synuclein[1]. Thus, PD is considered a synucleinopathy. Clinical diagnosis of PD is straightforward in typical cases. However, it can be problematic in patients with atypical clinical features. Accuracy of clinical diagnosis of PD is suboptimal, since as many as 25% of patients can be misdiagnosed[2].

Progressive supranuclear palsy (PSP), corticobasal degeneration (CBD), multiple system atrophy (MSA) and dementia with Lewy bodies (DLB) are the most common causes of atypical Parkinsonism. All of these diseases manifest with Parkinsonism, which is poorly or only transiently responsive to dopaminergic treatment.

MSA is a synucleinopathy, like PD. Its pathologic hallmark is glial cytoplasmic inclusions, which consist of abnormal α-synuclein deposition in oligodendrocytes[3]. DLB is also a synucleinopathy, characterized by predominantly cortical Lewy bodies[4]. PSP and CBD on the other hand are considered tauopathies, since their main pathologic findings (tufted astrocytes and astrocytic plaques, respectively) consist of abnormal tau protein aggregates in astrocytes[5,6].

Tau protein can present in six isoforms, depending on the alternate splicing of the microtubule associated protein tau (*MAPT*) gene[7]. This results in the variable expression of no, one or two oligonucleotides (N1 and N2) coded by exons 2 and 3 of the *MAPT* gene, as well as the presence of 3-repeat (3R) or 4-repeat (4R) microtubule binding regions coded by exon 10. Depending on the predominance of 3R- or 4R-tau isoforms, tauopathies are further divided into 4R- or 3R-tauopahies. PSP and CBD are considered 4-repeat (4R) tauopathies, Alzheimer's disease (AD) is a mixed 3R- and 4R-tauopathy, whereas Pick's disease is a 3R-tauopathy.

Total tau protein (τ_T), phosphorylated tau protein at threonine 181 ($\tau_{P\text{-}181}$) and amyloid beta with 42 amino acids ($A\beta_{42}$) are well-characterized cerebrospinal fluid (CSF) biomarkers of AD. These biomarkers have been incorporated into the most recent AD diagnostic criteria[8] and are the basis of the recently proposed AT(N) taxonomy system, which introduces biomarkers (according to their molecular specificity) for the *in vivo* pathological characterization of patients with AD[9].

The aim of this review is to present data on the utility of these three classical CSF biomarkers (τ_T, $\tau_{P\text{-}181}$ and $A\beta_{42}$) in the differential diagnosis of atypical Parkinsonism from PD. To this end, only studies which included patients with atypical Parkinsonism *vs.* PD or healthy controls are included.

This review includes representative studies which either have established our current knowledge on CSF biomarkers in Parkinsonism or provide new insights on the subject [Table 1].

STUDIES IN PSP

Most studies do not report any differences in CSF $A\beta_{42}$ between PSP and other causes of Parkinsonism or controls[10-16]. Interestingly, however, some studies have reported lower $A\beta_{42}$ values in PSP compared to controls[17-19]. Moreover, a single study reported lower $A\beta_{42}$ levels in PSP *vs.* PD[19]. According to this study, $A\beta_{42}$ could differentiate PSP from PD with 83% sensitivity and 67% specificity.

Regarding CSF τ_T, several studies could not establish any differences between PSP patients and controls[13-17]. Likewise, no difference in τ_T was evident between PSP and CBD[13,15-17], MSA[13,15,16] or PD[15,16] in most studies. Two studies have supported that PSP patients exhibit lower τ_T levels when compared to

Table 1. Overview of the studies with data on classical CSF biomarkers in cohorts with Parkinsonism

	$A\beta_{42}$	τ_T	$\tau_{P\text{-}181}$	Comparison group
Studies in PSP				
Holmberg *et al*.[10]	↔			Ctrl, MSA, PD
Verbeek *et al*.[11]	↔			Ctrl, MSA, PD
Aerts *et al*.[12]	↔[a]	↓[b] ↑[c]	↔[a]	a. CBD, PD, ctrl b. CBD c. PD
Hall *et al*.[13]	↔[a] ↑[b]	↔[c]	↓[d]	a. MSA, CBD, PD, PDD, ctrl b. DLB c. ctrl, CBD, MSA d. ctrl
Schoonenboom *et al*.[14]	↔	↔	↔	DLB, CBD, ctrl
Magdalinou *et al*.[15]	↔	↔	↔	CBD, MSA, PD, ctrl
Constantinides *et al*.[16]	↔	↔	↔	MSA, PD, CBD, ctrl
Noguchi *et al*.[17]	↓[a]	↔[b]	↔[b]	a. ctrl b. ctrl, CBD
Wagshal *et al*.[18]	↓	↓	↔	ctrl
Schirinzi *et al*.[19]	↓	↓	↓	ctrl
Süssmuth *et al*.[20]	↔	↔	↔	MSA, PD
Studies in CBD				
Aerts *et al*.[12]	↔[a]	↑[a]	↑[b] ↔[c]	a. ctrl, PSP, PD b. PD c. ctrl, PSP
Constantinides *et al*.[16]	↓[a]	↑[b] ↔[c]	↔[d]	a. PD b. ctrl c. PSP, MSA, PD d. PSP, MSA, PD, ctrl
Mitani *et al*.[21]		↑		ctrl
Urakami *et al*.[22]		↑		ctrl, PSP
Urakami *et al*.[23]		↑		ctrl, PSP
Hall *et al*.[13]	↔[a] ↑[b]	↑[c] ↔[d]	↔[e] ↑[f]	a. MSA, PSP, PD, PDD, ctrl b. DLB c. PD d. PSP e. PSP, PD, ctrl f. MSA
Schoonenboom *et al*.[14]	↓	↔	↔	ctrl
Magdalinou *et al*.[15]	↔	↔	↔	MSA, PSP, PD, ctrl
Noguchi *et al*.[17]	↔[a] ↓[b]	↔[c]	↔[c]	a. PSP b. ctrl c. ctrl, PSP
Studies in MSA				
Holmberg *et al*.[10]	↓			PD, PSP, ctrl
Shi *et al*.[26]	↓[a] ↔[b]	↔[c]	↔[c]	a. ctrl b. PD c. ctrl,PD
Verbeek *et al*.[11]	↔			Ctrl, PD
Hall *et al*.[13]	↔[a] ↑[b]	↑[c]	↓[d]	a. MSA, CBD, PD, PDD, ctrl b. DLB c. PD, PDD d. PD, ctrl
Magdalinou *et al*.[15]	↔	↔	↔	CBD, PSP, PD, ctrl
Constantinides *et al*.[16]	↔	↔	↔	PSP, CBD, PD, ctrl
Süssmuth *et al*.[20]	↔[a]	↑[b]	↔[c]	a. PD, PSP b. ctrl, PD c. PSP, PD, ctrl
Mollenhauer *et al*.[27]	↔	↔		PSP, PD
Abdo *et al*.[28]		↑		ctrl
Herbert *et al*.[29]		↑		Ctrl, PD
Abdo *et al*.[30]		↑		PD
Herbert *et al*.[31]		↑		PD
Studies in DLB				
Schoonenboom *et al*.[14]	↓[a] ↑[b]	↓[b] ↑[a]	↓[b] ↑[a]	a. ctrl b. AD

Mollenhauer *et al*.[32]	$\downarrow^a \leftrightarrow^b$	$\downarrow^b \leftrightarrow^a$	$\uparrow^a$	a. ctrl b. AD
Mollenhauer *et al*.[33]	$\downarrow^a \leftrightarrow^b$	$\downarrow^b \uparrow^a$	$\leftrightarrow^b$	a. ctrl b. AD
Mulugeta *et al*.[34]	$\downarrow^a \leftrightarrow^b$	$\leftrightarrow^c$	$\leftrightarrow^c$	a. ctrl b.AD, PDD c. ctrl, PDD
Llorens *et al*.[35]	$\downarrow^a$	$\uparrow^b$		a. ctrl, PD, PDD b. PD, MSA
Van Steenoven *et al*.[36]	$\downarrow$			ctrl
Parnetti *et al*.[37]	$\downarrow^a \leftrightarrow^b$	$\downarrow^b \uparrow^c$	$\downarrow^b$	a. PD, PDD b. AD c. PD
Kaerst *et al*.[38]	$\downarrow$	$\uparrow$		PD
Gmitterova *et al*.[39]	$\downarrow$	$\uparrow$		PDD
Gómez-Tortosa *et al*.[40]	$\uparrow^a \leftrightarrow^b$	$\downarrow^a \leftrightarrow^b$		a. AD b. ctrl
Boström *et al*.[41]	$\uparrow^a$	$\downarrow^a$	$\uparrow^b$	a. AD b. ctrl
Abdelnour *et al*.[42]	$\uparrow$			ctrl
Mollenhauer *et al*.[43]		$\leftrightarrow$		PDD

↑: elevated CSF levels; ↓: decreased CSF levels; ↔: no difference in CSF levels; CSF: cerebrospinal fluid; AD: Alzheimer's disease; PD: Parkinson's disease; MSA: multiple system atrophy; PSP: progressive supranuclear palsy; CBD: corticobasal degeneration; DLB: dementia with Lewy bodies; PDD: parkinson's disease dementia

controls[18,19], whereas a single study posited that patients with PSP have lower τ_T levels compared to CBD, but higher τ_T levels compared to PD[12].

CSF $\tau_{P\text{-}181}$ does not seem to be useful in the differentiation of PSP from other causes of Parkinsonism or controls[12,14-18]. Two studies have reported lower $\tau_{P\text{-}181}$ levels compared to controls[13,19].

Few studies include data on ratios of CSF biomarkers. In a large study, which included PSP, CBD and AD patients, elevated $\tau_{P\text{-}181}/A\beta_{42}$ ratio values could differentiate AD from PSP and CBD[17]. Another study posited that lower $\tau_{P\text{-}181}/\tau_T$ ratio values could discriminate patients with atypical Parkinsonism (PSP and MSA) from PD[20]. This ratio was optimal for discriminating PSP from CBD with a reported sensitivity of 86% and specificity of 75%[12]. PSP patients could also be differentiated from controls by lower $A\beta_{42}/\tau_T$[12] and higher $\tau_T/\tau_{P\text{-}181}$[16] ratios.

A single study examined classical CSF biomarkers in different phenotypes of PSP. To this end, patients with classic Richardson's syndrome (RS) were compared to patients with PSP-Parkinsonism (PSP-P). Interestingly, only patients with PSP-P had elevated total τ_T levels, compared to RS, PD and controls. $A\beta_{42}$ on the other hand was significantly lower in RS patients compared to PSP-P[20].

Few PSP patients seem to harbor a CSF-AD profile (generally defined as decreased $A\beta_{42}$ with elevated τ_T or $\tau_{P\text{-}181}$). In a large cohort, including diverse neurodegenerative disorders, 10% of PSP patients had a CSF-AD profile, as determined by an index incorporating CSF $A\beta_{42}$ and $\tau_{P\text{-}181}$ values[14]. Likewise, only one of 19 PSP patients (~5%) had a typical CSF-AD profile, as determined by abnormal $A\beta_{42}$, τ_T and $\tau_{P\text{-}181}$ values, in a cohort of patients with Parkinsonism[16].

Lastly, the possible relationship between CSF biomarkers and clinical characteristics has also been extensively studied in PSP. Most studies agree that there is no clinical-biochemical correlation in PSP[12-15,17]. Two studies have correlated low $A\beta_{42}$ levels with higher disease severity, as measured by Hoehn and Yahr score[10] or the PSP Rating Scale[19].

As a general conclusion, there are no significant abnormalities in the classical CSF biomarkers in PSP. Despite being a 4R-tauopathy, CSF tau proteins do not seem to differ between PSP and other Parkinsonian disorders. CSF total tau protein, which is considered a non-specific marker of neurodegeneration, would be expected to increase in PSP, as is the case in AD. A plausible explanation would be that tau protein in PSP is concentrated intracellularly (in astrocytic plaques, tufted astrocytes and neurofibrillary tangles), and for this reason it does not enter the cerebrospinal fluid.

Additionally, no obvious clinical-biochemical correlation has been observed, while an AD biomarker profile in PSP (indicating an AD co-occurrence or possibly a PSP-like phenotype of AD) is a rarity. The presence of a CSF-AD profile in PSP patients is more likely to be indicative of the presence of dual underlying pathologies (AD and PSP), since pathological studies have not established a correlation of AD pathology and Richardson syndrome (the most common phenotype of PSP).

STUDIES IN CBD

Most studies have focused on CSF tau proteins in CBD, since CBD is a tauopathy. Several of these studies have reported elevated levels of τ_T in CBD, compared to controls[12,16,21-23], PSP[12,22,23] and PD[12,13]. Interestingly, τ_T could differentiate CBD from PSP with 80% specificity and sensitivity in one study[23]. Likewise, CSF τ_T provided 75% sensitivity and 90% specificity in the discrimination of CBD from PD in another study[12]. However, no difference in τ_T between CBD and controls[14,15,17], PSP[13,15,16,20], MSA[15,16] and PD[15,16] has also been reported.

Regarding CSF $\tau_{P\text{-}181}$, most studies did not establish any meaningful difference between CBD patients and other Parkinsonian disorders or controls[13-17]. A single study has reported elevated CSF $\tau_{P\text{-}181}$ levels in CBD compared to PD[12], and another study in CBD patients compared to MSA patients[13].

CSF $A\beta_{42}$ levels do not differ in CBD compared to other Parkinsonian disorders, according to several studies[12,13,15,17]. However, lower $A\beta_{42}$ has been described in CBD patients compared to controls[14,17] and PD[16].

Regarding CSF biomarker indices, few studies have included relevant data in CBD. One study reported decreased $A\beta_{42}/\tau_T$ ratio in CBS compared to PD and controls, whereas $\tau_{P\text{-}181}/\tau_T$ ratio in CBS was decreased compared to controls[12]. According to this study, $\tau_{P\text{-}181}/\tau_T$ was optimal for PSP *vs.* CBD discrimination (86% sensitivity and 75% specificity). Another study posited that CBD patients have elevated $\tau_T/A\beta_{42}$ and $\tau_{P\text{-}181}/A\beta_{42}$ compared to PD[16].

Clinical and demographic data do not correlate with levels of classical CSF biomarkers in CBD, according to most relevant studies[13-15]. A single study reported that cognitive status, as measured by MMSE, correlated with both τ_T and $\tau_{P\text{-}181}$ levels and was inversely correlated with $A\beta_{42}$ levels[12]. Intriguingly, τ_T levels in CBD and PSP were dependent on disease severity in a single study, with maximal levels in medium-stage disease[23].

Few studies have focused on the presence of a CSF-AD profile in cohorts of Parkinsonian disorders. According to this approach, patients are divided in those harboring a CSF-AD profile and those who do not harbor such a profile. An initial study concluded that 20% of CBS patients harbored a CSF-AD profile (as defined by $\tau_T > 400$ pg/mL, $A\beta_{42} < 400$ pg/mL and $\tau_T/A\beta_{42}$ ratio > 1)[24]. In another cohort, 38% of CBS patients had a CSF-AD profile, based on an index which included $\tau_{P\text{-}181}$ and $A\beta_{42}$ values[14]. Along the same lines, a third study posited that ~30% of CBS patients had a typical CSF-AD profile (elevated τ_T and $\tau_{P\text{-}181}$ combined with decreased $A\beta_{42}$)[16]. Interestingly, when patients with a CSF-AD profile were excluded from analyses, an initially reported elevated τ_T and decreased $A\beta_{42}$ protein in CBS disappeared, and CBS patients

no longer differed from PD or controls. This implied that the elevated τ_T and decreased $A\beta_{42}$ levels in CBS might have been the result of the inclusion of AD patients who presented with a CBS phenotype. The CSF profile was implemented in another study by the same study group to investigate possible differences between AD and non-AD pathology in a CBS cohort[25].

In conclusion, abnormalities in classical CSF biomarkers are common in CBD. The commonly reported elevation in τ_T and $\tau_{P\text{-}181}$ and decrease in $A\beta_{42}$, does not seem to be a biochemical fingerprint of CBD but rather is a result of the inclusion in analyses of patients with AD pathology and a CBS phenotype. Up to 30% of CBS patients have an AD-biomarker profile, which is in accordance with pathological studies. Thus, CSF biomarkers are particularly useful tools in the *in vivo* discrimination of corticobasal syndrome in CBS-non-AD and CBS-AD.

STUDIES IN MSA

An initial study on CSF $A\beta_{42}$ in MSA reported lower levels compared to PD, PSP and controls[10]. A different study group also found lower $A\beta_{42}$ levels in MSA compared to controls[26]. However, several other studies could not establish any meaningful difference in $A\beta_{42}$ levels in MSA compared to other Parkinsonian disorders or controls[11,13,15,16,20,26,27].

Results regarding τ_T in MSA are conflicting, with most studies reporting an increase of τ_T in MSA compared to either controls[20,28,29] or other Parkinsonian disorders[13,20,29-31]. However, some studies have reported no difference between MSA and other Parkinsonian disorders[16,26,27], whereas a single study has supported that MSA patients exhibit lower τ_T compared to controls[26].

Few studies have included $\tau_{P\text{-}181}$ data in the comparison of biomarkers in MSA and other Parkinsonian disorders. These studies did not find any difference in $\tau_{P\text{-}181}$ levels in MSA compared to other study groups[15,16,20,26]. A single study has supported lower $\tau_{P\text{-}181}$ levels in MSA compared to PD and controls[13].

Two studies have included CSF biomarker indices in the differential diagnosis of MSA from related disorders. More specifically, a study has supported that MSA patients have significantly lower $\tau_{P\text{-}181}/\tau_T$ ratios compared to PD[20]. Another study posited that higher values of $\tau_T/A\beta_{42}$ ratio could differentiate MSA from PD with high specificity but only moderate sensitivity[16].

Regarding disease subtypes, MSA-C and MSA-P patients did not differ in their CSF biochemical profile in all relevant studies[11,20,28,30].

Clinical-biochemical correlation studies in MSA are sparse. A study correlated disease severity in MSA with lower CSF $A\beta_{42}$ levels[10]. Another study supported that both τ_T and $\tau_{P\text{-}181}$ levels increased with age in MSA[26]. A study implementing a battery of CSF biomarkers in a cohort of diverse neurodegenerative disorders could not establish any correlation between CSF biomarkers and clinical characteristics[15]. In conclusion, data on classical CSF biomarkers are largely inconclusive.

STUDIES IN DLB

It is well documented that CSF $A\beta_{42}$ in DLB is decreased compared to controls[14,32-36], PD[35,37,38] and parkinson's disease dementia (PDD)[35,37,39] according to the majority of studies on the subject. Comparison of $A\beta_{42}$ levels between AD and DLB has yielded conflicting results, with some studies reporting greater $A\beta_{42}$ levels in DLB compared to AD[14,40,41], and other studies not reporting any significant difference between the two groups[32-34,37]. A single study reported greater $A\beta_{42}$ values in DLB compared to controls[42] and another study did not find any difference between the two groups[40]. Another study did not report a difference between DLB and PDD[34].

There is consensus among studies that CSF τ_T is increased in AD compared to DLB[14,32,33,37,40,41]. DLB patients have been reported to harbor elevated τ_T levels compared to controls[14,33], although several studies could not establish any difference between DLB and controls[32,34,40]. Regarding other synucleinopathies (PD, PDD and MSA), studies have produced mixed results, reporting either an increase in τ_T in DLB compared to PD/PDD[35,37-39] and MSA[35] or no difference among patient groups[34,43].

Few studies have included τ_{P-181} in analyses of DLB cohorts. According to these limited studies, τ_{P-181} is elevated in AD compared to DLB[14,37], although one study could not establish a difference between AD and DLB[33]. Comparison between DLB and controls regarding τ_{P-181} has produced mixed results, with some studies reporting an increase in τ_{P-181} in DLB[14,32,41], whereas other studies could not establish any difference between groups[34]. PD/PDD patients do not differ from DLB in their τ_{P-181} profile[34,44].

Lower CSF $A\beta_{42}$ levels in DLB correlate with a worse outcome, according to several studies[14,40,42,45]. Likewise, elevated τ_T levels correlate with a poorer prognosis in DLB[37-39,41].

Several studies in DLB, which contain longitudinal data regarding CSF biomarker level alterations over time, provide conflicting results. Two studies have reported stable $A\beta_{42}$ over time in cohorts of DLB patients[39,46]. According to these studies, CSF τ_T and τ_{P-181} levels either increase[39] or decrease over time[46]. Conversely, two other studies have provided a different profile, with unaltered τ_T and τ_{P-181} levels over time, combined with a decrease in $A\beta_{42}$ during the transition from prodromal to demented stage of DLB[47,48].

A CSF-AD profile (as defined by a decrease in $A\beta_{42}$ and an increase in τ_T and/or τ_{P-181}) is common in DLB patients, presenting in 25%-50% of patients[14,42,45,49]. This profile invariably correlates with poorer prognosis[42,45]. According to a recent study, as many as 85% of DLB patients have decreased $A\beta_{42}$ in CSF, either isolated (45%) or in the context of a CSF-AD profile (40%)[49].

A single study has provided insight into the relationship between pathological finding and CSF biochemical profile[44]. In this cohort, 72% of DLB patients had senile plaques and 50% had neurofibrillary tangles. Interestingly, there was a correlation between the presence of senile plaques and CSF $A\beta_{42}$ levels. The co-occurrence of neurofibrillary tangles did not affect τ_T and τ_{P-181} levels in DLB patients.

In conclusion, there is a general agreement that $A\beta_{42}$ levels are decreased in DLB, either alone or accompanied by increased τ_T or τ_{P-181} levels. The decrease of $A\beta_{42}$ levels have been shown to correlate with faster disease progression. Decreased CSF $A\beta_{42}$ levels and increased τ_T and τ_{P-181} in DLB could be attributed to the frequent co-occurrence of AD pathology in patients with Lewy body disease. This hypothesis would explain the correlation of a CSF-AD profile with worse outcomes in DLB patients. Intriguingly, multiple lines of evidence suggest an interplay between α-synuclein and tau/amyloid beta aggregation, which may explain the frequent co-occurrence of AD pathology and Lewy body disease[50-52].

METHODOLOGICAL CONSIDERATIONS

Most studies on classical biomarkers in atypical Parkinsonism implemented enzyme-linked immunosorbent assays (ELISAs). Advantages of ELISAs include relatively low cost, high reproducibility and high availability, since commercial ELISAs of classical CSF biomarkers are readily available. There have been efforts, however, to implement novel techniques and to target different proteins in search of new biomarkers in neurodegenerative diseases.

To this end, a study examined differences in tau isoforms in CSF, which may reflect differences in post-translational processing of tau protein. More specifically, differences in tau proteolytic products in CSF were the main study endpoint. By means of immunoprecipitation, extended (55 kDa) and truncated forms

(33 kDa) of tau protein were determined in CSF. The tau 33 kDa/55 kDa ratio was significantly lower in PSP patients, compared to patients with AD, FTD, CBD, PD and DLB and emerged as a possible biomarker for PSP[53]. The same study group replicated these results in a larger cohort[54]. However, these promising results were not be replicated by another study group[55].

In another study, immuno-PCR essays were developed to measure 3R- and 4R-tau isoforms in CSF. These essays were tested in four different cohorts, which included PSP, CBD, PDD and PDD patients. The study we are referring to included four cohorts, in which the 3R and 4R-tau isoform levels varied considerably among the four cohorts (mean values in control groups ranging for both 3R- and 4R-tau from < 5 pg/mL to ~50 pg/mL). Analysis of tau isoforms in all cohorts combined indicated lower 4R-tau levels in PSP and AD. 3R-tau did not differ among study groups. The 3R-tau/τ_T and 3R-tau/$\tau_{P\text{-}181}$ ratios were decreased in AD compared to PDD. The 4R-tau/τ_T was lower in PSP and AD compared to controls and the 4R-tau/$\tau_{P\text{-}181}$ ratio was higher in PDD compared to PSP, CBD and AD[56].

Wagshal *et al.*[18] developed a multitude of novel ELISAs, which target different epitopes of tau proteins, in order to examine differences in the concentration of diverse tau protein fragments in CSF. These ELISAs were applied in patients with AD, PSP and controls. Interestingly, PSP patients had lower concentrations of most tau fragments compared to controls.

Another study group implemented mass spectrometry to quantify tau-specific peptides in the entire sequence of tau protein in a cohort of PSP, AD and DLB. This novel approach yielded data for 18 tau peptides. By use of these peptides, the authors determined that the 1N and 3R-tau isoforms were mostly represented in CSF. Levels of tau peptides were intra-correlated and significantly increased in AD patients. Interestingly, AD patients had relatively decreased levels of peptides in the central core region of tau protein. This region contains phosphorylation sites, which may explain this finding[57].

Along the same lines, Cicognola *et al.*[58] developed novel antibodies against tau fragments at amino acid 123 and 224 (tau-123 and tau-224, respectively), after having identified endogenous tau fragments ending at these amino acids. They concluded that anti-tau-224, but not anti-tau-123, was present in neurofibrillary tangles and neuropil threads of AD patients, whereas tau-224 levels were elevated in CSF of these patients and correlated with conversion from mild cognitive impairment to AD. The authors concluded that only tau-224 is neuron-specific, whereas tau-123 may represent a general non-specific marker of tau metabolism.

DISCUSSION

There has been extensive research on classical CSF biomarkers in patients with Parkinsonism, as illustrated in this review of the literature. Most studies agree that PSP patients do not exhibit a specific CSF biochemical profile. Few studies have reported decreased $A\beta_{42}$ levels. A CSF-AD profile has rarely been described in PSP patients.

An elevation in CSF τ_T levels has been systematically reported in CBS. Moreover, few studies have also documented a decrease in $A\beta_{42}$ and an increase in $\tau_{P\text{-}181}$ in these patients. Interestingly, a significant percentage (< 40%) of CBS patients can harbor a CSF-AD profile[14,16,24]. This is in agreement with clinical-pathological studies of CBS[59-62], where AD is a relatively common underlying pathology of CBS. A study emphasized that the frequently reported elevation in CSF τ_T and reduction in $A\beta_{42}$ in CBS might not represent an inherent biochemical characteristic of CBS and could be attributed to the inclusion of AD patients presenting with CBS[16]. Thus, classical CSF biomarkers are particularly important in CBS cohorts, since they can indicate an underlying AD pathology.

Regarding MSA, most studies report an elevation in CSF τ_T protein, whereas a decrease in $A\beta_{42}$ levels in MSA has been rarely reported. Although an interplay between α-synuclein and $A\beta_{42}$ has been established

in vitro, alterations in CSF $A\beta_{42}$ levels in a synucleinopathy are difficult to explain[63]. In a transgenic mouse model with expression of both β-amyloid peptides and synuclein, β-amyloid peptides promoted aggregation of α-synuclein[50]. Likewise, pathological data from PD and DLB patients support the accumulation of tau oligomers in these brains. Moreover, oligomeric tau forms may co-exist in the same aggregates with α-synuclein, forming hybrid oligomers[51]. Along the same lines, α-synuclein may induce specific toxic tau oligomers in cell cultures[52].

DLB patients exhibit significantly lower CSF $A\beta_{42}$ values compared to other Parkinsonian disorders. Moreover, studies support that τ_T in DLB may be elevated. However, AD can be differentiated from DLB by means of a significant increase in τ_T and $\tau_{P\text{-}181}$ in AD compared to DLB. A significant percentage of DLB patients harbor a CSF-AD profile, which indicates the frequent co-occurrence of Lewy body and AD pathologies, especially in older patients.

CONCLUSIONS AND FUTURE DIRECTIONS

Classical CSF biomarkers in atypical Parkinsonism are important, particularly for the diagnostic work-up of CBS and DLB patients. A CSF-AD profile in a CBS patient indicates an underlying AD pathology. Conversely, in DLB patients, the presence of a CSF-AD profile indicates the co-occurrence of AD and Lewy body pathologies, which usually correlates with poorer prognosis. Although some differences have been reported in classical CSF biomarkers in both PSP and MSA, the results are inconsistent and require further research.

The disparity of results on classical biomarkers in atypical Parkinsonism can be largely attributed to the great heterogeneity of studies. This heterogeneity refers to diagnostic criteria, cohort synthesis, statistical analysis and pre-analytical and analytical factors. Standardization of these pre-analytical and analytical confounders, as established in recent recommendations, is paramount for more robust results[64]. Moreover, large cohorts of Parkinsonian disorders with CSF biomarker data and pathology-confirmed diagnoses are lacking. These studies would reliably inform us on the interplay between CSF biomarkers and pathological findings. This disparity in results also illustrates the limitations of classical biomarkers in the differential diagnosis of patients with Parkinsonism, and emphasizes the need for novel approaches.

To this end, differences in isoforms of these known proteins (e.g., 3R- *vs.* 4R-tau protein), differences in proteolytic products and quantification of specific peptides of these proteins are promising new approaches. Moreover, better characterization of other proteins which may serve as biomarkers (such as α-synuclein or TDP-43) is of great importance.

DECLARATIONS

Authors' contributions

Conception of the study, drafting of the manuscript, literature review: Constantinides VC
Conception of the study, critical appraisal of manuscript: Paraskevas GP, Boufidou F
Conception of the study, critical appraisal of manuscript, literature review: Bourbouli M, Paraskevas PG
Conception of the study, critical appraisal of manuscript: Stefanis L, Kapaki E

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All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

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Neuroimmunology and Neuroinflammation

Review

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Assessment of cerebrospinal fluid α-synuclein as a potential biomarker in Parkinson's disease and synucleinopathies

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Abstract

The discovery of diagnostic and prognostic biomarkers for neurodegenerative diseases represents an unmet clinical challenge. For example, the diagnosis of Parkinson's disease (PD) relies mainly on the presence of clinical symptoms. Therefore, the identification and use of novel PD biomarkers would allow the application of disease-modifying treatments at the very early stages of neurodegeneration. The presynaptic protein, α-synuclein, has been genetically and biochemically linked with PD pathogenesis and has been considered as a potential biomarker for the diagnosis of PD and the related synucleinopathies. The vast majority of studies have assessed the measurement of α-synuclein, alone or in combination with other biomarkers in the cerebrospinal fluid (CSF), since it is the biofluid that most closely reflects the pathophysiology of the brain. The diagnostic value of the monomeric α-synuclein but also the oligomeric, the phosphorylated and the aggregated forms of the protein has been evaluated using a variety of immunoassays. The results have so far been reproducible but the assays used are still lacking the required diagnostic accuracy. Recent reports have shown that Protein misfolding cyclic amplification is a technique that has the potential to detect α-synuclein seeds in samples of CSF with high sensitivity and across different synucleinopathies. In an effort to increase the source of biomarker for PD and related synucleinopathies, α-synuclein has also been measured in neuronal exosomes, small vesicles of endosomal origin that are secreted from neurons into the CSF or the periphery. The potential diagnostic value of exosomes stems from the notion that exosomes carry a disease-specific repertoire of marker proteins. Therefore, the assessment of exosome-associated α-synuclein species may also open up new avenues for disease diagnosis in different synucleinopathies.

Keywords: Cerebrospinal fluid, α-synuclein, Parkinson's disease, biomarker, exosomes, synucleinopathies

INTRODUCTION

The formation of large inclusions mostly containing protein aggregates is a common pathological hallmark in a wide spectrum of neurodegenerative disorders such as Alzheimer's Disease (AD) and Huntington Disease[1]. Particularly three distinct neurological conditions, Parkinson's Disease (PD) including Parkinson's disease dementia, Dementia with Lewy Bodies (DLB) and Multiple System Atrophy (MSA), are characterized by the aberrant accumulation of the presynaptic protein α-synuclein. In PD and DLB, α-synuclein deposits are found either in the cytoplasm of neurons, where they are called lewy bodies (LB), or in the neuronal terminals, where they are called Lewy neurites[2] whereas in MSA, α-synuclein deposition occurs in glial cells. α-Synuclein is also genetically linked with the development of PD since specific point mutations or multiplications (duplications, triplications) of the *SNCA* gene encoding for α-synuclein result in the familial forms of PD[3-8]. The genetic association of α-synuclein with PD is further strengthened by all the genome-wide association studies performed so far which indicate a strong correlation of PD with variations in the *SNCA* gene[9,10]. These biochemical and genetic linkages of α-synuclein with pathology as well as the observation that the protein is present in biological fluids or peripheral tissues led to the assumption that α-synuclein could serve as a potential candidate biomarker for PD diagnosis and also aid the differential diagnosis between the synucleinopathies[11].

A plethora of studies have assessed the absolute quantification of α-synuclein levels as a marker of synucleinopathy with the ultimate aim to discriminate PD patients from healthy subjects or other unrelated neurological controls. In this regard, understanding the structural biology of α-synuclein is critical; the protein is highly modified at the post-translational level and has the ability to adopt different conformations depending on the surrounding milieu. From all the modifications that have been reported so far, phosphorylation is considered most closely related to PD pathology since almost 90% of α-synuclein in LB appears to be hyper-phosphorylated[12]. In addition, the assembly into multiple-sized oligomers has been considered an early event in the pathological process of aggregate formation[13]. As such, different forms of α-synuclein, i.e., monomeric, oligomeric and phosphorylated, have been targeted in order to increase diagnostic accuracy[14]. The measurements have been performed in bodily fluids [cerebrospinal fluid (CSF), blood plasma or serum, saliva], isolated secreted vesicles (exosomes) and peripheral tissues (skin, olfactory or gut mucosa, salivary gland) using a variety of analytical approaches depending on the nature of the biological sample and the form of α-synuclein detected with each assay[15].

In comparison with the other biological fluids, the assessment of α-synuclein in the CSF has provided the most consistent results in terms of analytical validation by different laboratories[16]. CSF α-synuclein is mostly detected by means of immunoassays that use specific antibodies to target the different α-synuclein forms. Even though the absolute concentrations can vary from study to study, the results obtained so far are supported by several meta-analysis studies suggesting that CSF α-synuclein could serve as a potential marker of synucleinopathy[17]. In this review, we aim to discuss the results from the assessment of α-synuclein in CSF and exosomes and explain the factors responsible for the variability among the different studies.

MEASUREMENT OF CSF α-SYNUCLEIN

Being primarily produced by the choroid plexus within the ventricles of the central nervous system, CSF is an established biological fluid to study neurodegenerative disorders since it is expected to mirror brain microenvironment. The quantification of total α-synuclein, as well as its oligomeric and phosphorylated forms, can be measured in CSF using different techniques, such as ELISA[18], xMAP technology[19],

mass spectrometry[20], time-resolved fluorescence energy transfer[21], electrochemiluminescence immunoassay[22] and western blot[23]. In addition, new biochemical assays that can detect α-synuclein aggregates have emerged, such as Protein-misfolding cyclic amplification and Real-time Quaking-induced conversion, by taking advantage the ability of α-synuclein to nucleate further aggregation[24-26]. Using all these different methods, it is important to note that, even though there are variations in the absolute concentrations measured, the results produced for total (or monomeric) CSF α-synuclein agree on a reduction in α-synuclein levels in PD patients when compared with control subjects. When oligomeric or phosphorylated α-synuclein was assessed in the CSF, both forms were found to be increased in PD patients compared with the controls. However, it is important to note that the of ligands such as ThT may affect the actual structure of the α-synuclein species.

Even though the above findings are consistent, the diagnostic accuracy (sensitivity and specificity) remains unsatisfactory either for the detection of the monomeric or the modified forms of α-synuclein. Additionally, some studies report contradictory results; some have found similar CSF α-synuclein levels between PD patients and control subjects[27-29], whereas others have reported increased CSF α-synuclein levels in samples from AD[30,31], progressive supranuclear palsy or Creutzfeldt-Jacob patients compared with the control group[32].

A number of factors could explain the observed variability in the results, as well as the differences reported in the absolute concentrations. First, the immunoassays used are based on divergent antibodies that recognize different fragments of the protein and with variable affinity. Second, the patient cohorts show great variability in terms of number, disease stage and clinical symptoms (affected mobility or dementia) present at the time of CSF collection. Third, the implementation of strict standardized guidelines concerning collection and storage protocols and allowed blood contamination have only recently started to be followed. Furthermore, common reference materials are still missing making the interpretation of results from assay to assay extremely difficult.

The quantification of CSF α-synuclein could aid the differential diagnosis in clinically overlapping neurodegenerative diseases, as suggested for PD, DLB and AD[33-35]. However, it is unclear whether the levels of CSF α-synuclein could be correlated with the severity of disease, indicating for example a more rapid decline in motor performance or the appearance of dementia. Interestingly, recent reports have shown that simultaneous measurement of α-synuclein levels along with other proteins, such as tau, $A\beta_{42}$ and Glucocerebrosidase 1 could be more effective in discriminating PD patients with synucleinopathies from healthy individuals or those with other neurodegenerative diseases[30,36-38].

Other biological fluids could also serve as promising candidates for α-synuclein detection and subsequent PD diagnosis. The majority of reports studying plasma α-synuclein have exhibited increased levels in PD patients[39-46] relative to control subjects, whereas other studies have reported similar[27,47,48] or decreased plasma α-synuclein levels[49] between PD patients and healthy participants. Interestingly, it was found that plasma levels of phosphorylated α-synuclein were higher in the PD samples than the controls[44,50,51]. The results obtained from plasma have been controversial, mainly due to the fact that red blood cells are a major source of α-synuclein and the rest erythrocytes that remain in plasma[52] can be subjected to hemolysis markedly affecting α-synuclein values[47].

As erythrocytes are the major source of peripheral α-synuclein, a recent report has proposed erythrocytic α-synuclein as a potential PD biomarker, as it was found that the total and aggregated α-synuclein levels were significantly higher in the membrane fraction of PD patients compared to healthy controls[53]. Saliva α-synuclein has also been considered as a prospective biomarker, as α-synuclein pathology has been found in submandibular salivary glands[54,55] and saliva α-synuclein could be easily accessible and poorly affected

by blood contamination[56-59]. Some studies have reported that total α-synuclein levels were reduced in the saliva of PD patients compared with control subjects, whereas oligomeric α-synuclein appeared to be elevated in the saliva of PD patients[56,57].

MEASUREMENT OF α-SYNUCLEIN FROM NEURONAL EXOSOMES AS A POTENTIAL BIOMARKER

α-synuclein was considered to be localized mostly in the cytoplasm of neuronal cells, until several studies demonstrated its presence in human CSF, human plasma and in the conditioned medium of various cell lines[23,60]. Many studies have shown that α-synuclein is physiologically secreted in the extracellular space, but the mechanism of α-synuclein release is still unclear. Evidence from recent studies has also suggested that extracellular α-synuclein can confer to the progression of PD[1-3] and it has been proposed that α-synuclein secretion, either in a monomeric or oligomeric state, induces α-synuclein propagation via cell-to-cell transfer[61]. Thus, elucidating the mechanism by which α-synuclein is secreted in the extracellular space is of great importance in understanding cellular pathways that may cause PD.

Release of α-synuclein via extracellular vesicles termed exosomes has been demonstrated by our group and others[62,63]. Exosomes are extracellular vesicles of ~50 to 200 nm diameter and can mediate proximal and distal cellular communication through the transfer of biological molecules between cells. They originate from the inward budding of multi-vesicular bodies (MVBs) and are released to the extracellular space upon fusion of MVBs with the plasma membrane in an exocytic manner. Exosomes are released from numerous cell types including neurons and glia[64] and in several studies have been observed to be associated with pathologic proteins including α-synuclein[63,65]. Based on the current knowledge, exosomes are functionally active entities, with a highly versatile role, ranging from intercellular communication by delivering specific protein, lipid or RNA cargo, and removal of obsolete or misfolded proteins, as a means of cell detoxification, to deleterious shuttles that impair cell homeostasis[66].

Some well-characterized functions of exosomes are protein secretion and intracellular uptake, immune response regulation and toxicity induction[67]. Interestingly, Danzer *et al.*[65] demonstrated that exosome associated α-synuclein is more potent in transmitting aggregation pathology between neurons than free-secreted α-synuclein. One study has shown that patients with PD have higher α-synuclein levels in plasma exosomes compared to healthy controls[68], while Stuendl *et al.*[69] found decreased neural exosome α-synuclein levels in PD patients, consistent with the total α-synuclein levels in CSF. In addition, the quantification of CSF exosomal α-synuclein exhibited distinct differences between patients with PD and DLB. Moreover, exosomal α-synuclein levels correlated with the severity of cognitive impairment in cross-sectional samples from patients with DLB. In the same study, Stuendl *et al.*[69] showed that exosomes from PD and DLB patients contain pathogenic α-synuclein species which serve as seeds to induce the oligomerization of soluble α-synuclein in recipient cells. Shi *et al.*[70] have shown that CNS-derived exosomes can efflux into blood. Importantly, they found a substantially augmented α-synuclein concentration in the plasma-isolated exosomes from PD patients compared to healthy control subjects, despite the fact that no differences were detected in plasma total α-synuclein levels. Additionally, they report a significant increase of plasma exosomal α-synuclein/total α-synuclein ratio in PD patients, negatively correlated with the disease severity, further supporting the importance of the disease-related exosomal cargos as PD biomarkers with high sensitivity and specificity. The authors concluded that plasma, CNS-derived exosomal α-synuclein can serve as a PD biomarker with high sensitivity and specificity[70]. The same group has also shown that CNS-derived exosomal tau in plasma is significantly higher in PD patients than in controls and is correlated with CSF total tau and phosphorylated tau[71]. Furthermore, distinct circulating exosome entities have been identified in the serum of patients with PD[72]. A recent study demonstrated that the levels of DJ-1 and α-synuclein in plasma CSF-derived exosomes and the

ratio of plasma CSF-derived exosomal DJ-1 to total DJ-1 were significantly higher in patients with PD, compared with controls[4]. Several factors have been shown to affect the release of α-synuclein through exosomes such as the activity of Glucocerebrosidase enzyme (GCase), ion homeostasis, such as Zn^{2+}, Ca^{2+} and Mn^{2+}, as well as neuronal activity and neurotransmitter release. The heterozygous mutations in the *GBA1* gene are considered as an important risk factor for PD. In this regard, it has been demonstrated that GCase overexpression leads to a decrease of exosome secretion while chronic pharmacological inhibition of GCase activity *in vivo* profoundly increased exosomes levels, as well as exosome-associated α-synuclein oligomers[73]. In addition, decreased GCase activity has been demonstrated in brain samples with increased α-synuclein levels and in CSF from sporadic PD patients[74]. More recently, a study by Cerri *et al.*[75], showed that exosomes from PD patients contain a greater amount of α-synuclein compared to healthy subjects whereas no differences were found in plasma total α-synuclein levels. Importantly, the authors showed a significant inverse correlation between GCase activity and this ratio in PD patients.

Notably, exosomes being a snapshot of the intracellular milieu, comprise a great source of bioactive molecules, including various RNA species. In a study conducted by Gui and co-workers, where exosomal miRNAs were isolated from the CSF of PD patients, 16 and 11 exosomal miRNAs were found upregulated and down- regulated, respectively, in PD patients compared to controls[76]. Validated hits were found to be miR-1 and miR-19b-3p, significantly reduced in PD CSF exosomes, in contrast to miR-153, miR-409-3p, miR-10a-5p, and let-7g-3p which were found to be increased[76]. This evidence highlights the potential diagnostic value of CSF exosomal RNA in the assessment of PD.

Although the role of exosome-associated α-synuclein as a potential biomarker remains relatively controversial, there are specific parameters that should be taken into account. Firstly, with regards to the source of exosomes, including plasma, saliva, CSF, there are certain protocols for their acquisition, followed by even more meticulous procedures for exosome isolation and purification. Differences during the aforementioned protocols may account for deviations between groups. In the same context, total exosome isolation may mask differences that could be found in exosomes of specific cellular origin. For example, Tomlinson and colleagues[72], following an unbiased proteomic approach, did not find any significant increase of α-synuclein in total exosomes isolated by ultracentrifugation from the serum of PD patients. On the contrary, Shi *et al.*[70] showed a significant α-synuclein enrichment in neuron-specific exosomes, isolated from PD patients' plasma by immunoprecipitation of the neuronal adhesion molecule L1CAM. More importantly, the notion that α-synuclein exists and exerts its detrimental effects in different strains, leading to different aggregates that cause as many distinct synucleinopathies, i.e., PD, DLB, MSA has been cemented[77,78]. Given the fact that exosomal cargo mirrors the state of the cell from which it originates, exosome-associated α-synuclein may reflect the dynamic nature of α-synuclein species. To this end, it is of a pivotal importance to develop techniques that could allow detection and quantification of all the different α-synuclein conformers. Overall, although in its infancy, the study of exosome-associated α-synuclein as a potential biomarker is quite promising, yet, it requires more combinatorial approaches.

CONCLUSION

Over the last 10 years there has been considerable amount of research effort placed in the evaluation of neuronal α-synuclein as a diagnostic or, even, a prognostic biomarker for PD and related synucleinopathies. The majority of studies have indicated that CSF α-synuclein could be useful for the diagnosis of synucleinopathy that could also aid the distinguishment of PD patients from patients with other neurodegenerative conditions. However, its utility as a biomarker is hampered by the lack of a universally validated assay of high diagnostic accuracy. To this end, following the strict recently established standard operating protocols for CSF collection and storage and correlating the measurement of monomeric α-synuclein with the oligomeric or phosphorylated forms would greatly improve the diagnostic value of the assessment of α-synuclein in CSF. To further ameliorate the specificity of α-synuclein measurement, recent

experimental approaches involve the assessment of α-synuclein in neural exosomes. The discoveries of pathogenic misfolded proteins such as α-synuclein in them has generated intensive research into their use as biomarkers considering that they carry proteins with disease-specific fingerprints reflecting the presence and staging of the disease. However, in order to further verify this potential we need to have a very good understanding of the actual mechanisms behind their biogenesis and release. Importantly, we need to have those tools in place that will assist us in the identification of the α-synuclein species responsible for disease generation and pathology progression. The fact that exosomal cargo mirrors the state of the cell from which it originates[79] unravels the promising role of the plasma/CSF -derived exosomes as potential biomarkers. Proteomic profiling of exosomal proteins in PD patients with different disease stages and healthy subjects may also aid the identification of specific protein changes that occur in response to pathology progression. Finally, modulating exosome biogenesis and release may have a promising prospect in PD therapy.

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Authors' contributions

Contributed to the writing of the manuscript and in its revision: Chalatsa I, Melachroinou K, Emmanouilidou E, Vekrellis K

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COVID-19 neurotropism and implications for therapy

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Abstract

The mechanism underlying the pathogenesis of severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) infection in humans is poorly understood, although the cellular receptors which facilitate the virus fusion have been identified. Although the major symptoms of the infection have been identified as acute respiratory distress, pneumonia, and fever, recently, symptoms involving nervous system dysfunctions, including encephalopathy and stroke, have been detected. Herein, we comprehensively review the evidence that SARS-CoV-2 infection involves a neurotropic mechanism including a nose-brain-lung axis suggesting implications in therapy development.

Keywords: SARS-CoV-2, nose-brain-lung axis, neurotropism

INTRODUCTION

The current global pandemic outbreak of novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is known for its viral tropism to the lungs, which in severe cases can lead to the fatal respiratory failure of patients who have contracted the disease. SARS-CoV-2 and SARS-CoV viruses have a 70%-80% homology and both enter the body through the same receptor, angiotensin converting enzyme 2 (ACE2)[1].

In addition to their genetic homology, the pathology that the two viruses exhibit in the clinic are highly similar[2]. In a recent report from Wuhan, China, Mao and colleagues showed that in addition to respiratory symptoms including pneumonia and acute respiratory distress syndrome (ARDS), out of 214 hospitalized COVID-19 patients 78 (36.4%) showed neurological manifestations such as cerebrovascular diseases (5.7%), impaired consciousness (14.8%), and skeletal muscle injury (19.3%)[3]. Six percent of COVID-19 patients have been reported to show symptoms of stroke and 15% were reported to show encephalopathy[4]. A number of symptoms such as dizziness, headache, loss of taste and smell, impaired consciousness, seizures, and nerve pain suggest a neurological connection to this viral infection.

Previously, SARS-CoV was observed in the cerebrospinal fluid of a SARS patient showing ARDS[5]. Neuropathy in a COVID-19 patient has been reported in which the patient had hyposmia and altered taste sensation[6]. Establishing the link between prior coronaviruses and their neurotropism appears to be important because there is now mounting evidence for SARS-CoV-2 neuronal abnormalities emerging as a significant symptom associated with the disease[7]. COVID-19 patients can show neurological manifestation under three situations: due to neurotropism of the virus, post-infective neurological complications or aggravated symptoms in patients with neurological co-morbidities like dementia[8]. Moreover, the neurological complications and symptoms can be central, including headache, dizziness, altered sensorium, stroke, ataxia, encephalitis, and seizuers, or peripheral, like loss of smell or taste sensation or skeletal muscle injury[8]. Herein, we review a profound neurological basis of SARS-CoV-2 infection-induced global pandemic and how the viral infection modulates the breathing of the lungs leading to fatal ARDS, to which about 50%-80% of the severely ill patients succumb.

NEUROTROPISM OF β-CORONAVIRUSES

The family of β-coronaviruses, to which SARS and SARS-CoV-2 belong, have been extensively studied. Several of the β-coronaviruses have been discovered within the brain (especially the brain stem), including SARS-CoV, hCoV-229E, hCov-OC43, mouse hepatitis E, and porcine hemagglutinating encephalomyelitis (HEV) coronavirus[5,9]. Middle Eastern respiratory syndrome (MERS) virus, another notable zoonotic viral outbreak in 2008-2009, that utilizes a different host receptor, DPP4, has also been discovered in the brain stem of experimental animals even in the absence of lung infection[10], demonstrating a strong propensity for the family of β-coronaviridae to infect the brain. While ACE2 is expressed at low levels within the brain, it has been hypothesized that the "promiscuous" spike protein of β-coronaviruses may also have an unknown receptor in the brain and peripheral nerves to which the neurotropism is owed[10].

SARS-CoV and MERS-CoV are considered important coronaviruses with the potential of nervous system damage. SARS-CoV was found to cause neurological conditions like demyelination of nerve fibers, ischemic changes of neurons, and diseases such as encephalitis, polyneuropathy, and aortic ischemic stroke[11]. Similarly, 1 in 5 MERS-CoV patients were reported with neurological symptoms such as paralysis, ischemic stroke, loss of consciousness and Guillain Barré syndrome[12]. The presence of SARS-CoV has also been demonstrated in mice brain experimentally infected with SARS-CoV[13]. Another coronavirus, the mouse hepatitis virus type 3, can disrupt the BBB by down-regulating interferon β which would allow virus and infected immune cells in[14].

NEURAL CONTROL OF BREATHING

The major life-threatening symptoms of SARS-CoV-2 infection are respiratory failure and pneumonia. While pneumonia is largely an inflammatory condition of the lungs, breathing is extensively regulated by the brain. Breathing is regulated by numerous internal and external stimuli, which are integrated in the brain stem to produce the muscle movements necessary for inspiration (or inhalation), and expiration (or exhalation) [Figure 1A]. The rate and phases of the breathing pattern arise from respiratory centers

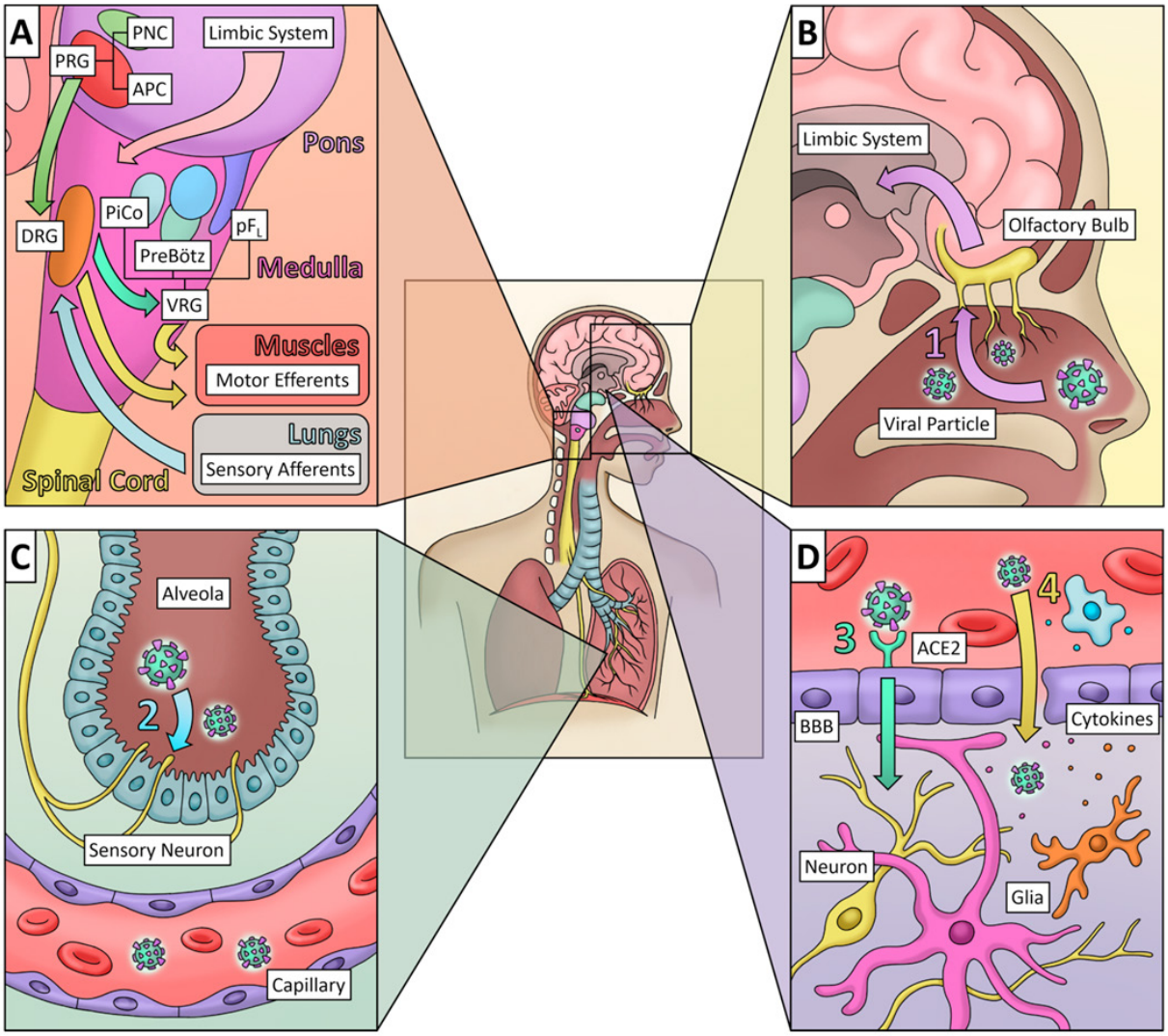

Figure 1. Respiratory centers of the brainstem and methods of viral entry into the nervous system. A: The brainstem contains the respiratory centers which integrate signals from the cortex through the limbic system and regulate breathing. Motor efferents from the brain stem innervate the diaphragm, airways, and abdominal muscles which create the movements necessary for inhalation and exhalation; B: olfactory neurons are susceptible to infection by viral particles in the nasal cavity (Route 1). These sensory neurons cross the cribiform plate and project through the olfactory tract into areas of the cortex and limbic system; C: alveoli in the lung are innervated by sensory neurons projecting up the spinal cord to the brainstem. The sensory neurons that innervate the lungs directly gather chemo- and mechano-signals from their dendrites in the lung and project them to pre-synapses in the brainstem. These neurons detect changes in oxygen and CO_2, mechanical stretch, and irritants that may be inhaled into the lung. Viral particles may enter these neurons and be transported anterogradely to the respiratory centers of the brainstem (Route 2). They may also enter the bloodstream and be subsequently introduced to the brain parenchyma through the blood–brain barrier (BBB) (Routes 3 and 4); D: a viral infection in the bloodstream may cross the BBB by active transport via receptors on the surface of endothelial cells (Route 3) or by passive diffusion through a leaky barrier (Route 4). Immune cells in the blood and glial cells in the brain respond to infection by releasing inflammatory cytokines, which may damage the BBB and result in greater passive diffusion of the virus. Arrows indicate the direction of signal transmission (A). Arrows indicate the direction of virus movement (B, C, D). Numerals 1-4 indicate different proposed routes of viral transmission. PNC: pneumotaxic center; APC: apneustic center; PRG: pontine respiratory group; pF$_L$: lateral parafacial nucleus; PiCo: postinspiratory complex; PreBötz: PreBötzinger complex; VRG: ventral respiratory group; DRG: dorsal respiratory group

in the pons and medulla. This control arises from neural circuits projecting from the cerebral cortex to the brainstem[15]. In addition to voluntary control of breathing, a variety of sensory pathways contribute to the breathing pattern[16]. Normal breathing consists of an inexorable active inspiration phase arising from rhythmic neural activity in the preBötzinger (preBötz) complex[17]. The preBötz is a region of the ventrolateral medulla which is active in phase with inspiration. Neurons in the preBötz project either

directly or indirectly to motor neurons which innervate the diaphragm, intercostals, and airway muscles [Figure 1A]. Rhythmic limb movement has also been shown to modulate breathing through afferent sensory pathways[18]. Of particular interest are vagal sensory neurons expressing chemoreceptors and mechanoreceptors, which project from the lungs and airways to respiratory centers of the brainstem[19]. Due to their inherent proximity to the primary infection site, these sensory neurons present one potential mechanism by which respiratory viruses may enter the brain[20]. Expiration is passive at rest. However, active expiration may be brought on by increased O_2 demand, such as respiratory distress[21]. Disinhibition or activation of neurons in the lateral parafacial nucleus (pF_L), which project to expiratory premotor neurons, causes contraction of muscles, which reduces lung volume below their resting capacity[22]. This reduced lung volume after active exhalation increases the volume of the subsequent breath resulting in greater O_2 delivery. A third phase, post-inspiration, may occur between the inspiration and exhalation phases. Post-inspiration is a delay of lung deflation which increases gas exchange in the lung[23]. It may originate from interneurons medial to the parafacial nucleus, referred to as the post-inspiratory complex (PiCo)[24]. Thus, infection of any of these regions of the brain may lead to respiratory distress or even failure.

EVIDENCE OF NEUROTROPISM OF SARS-COV-2

It has been postulated that, like other β coronaviruses, SARS-CoV-2 can also infect the brain by migrating from the general circulation to the cerebral microcirculation via endothelial cells which express ACE2[5]. In a recent case study published by Poyiadji *et al.*[25], they described an acute hemorrhagic necrotizing encephalopathy that was directly attributed to SARS-CoV-2 infection and the concurrent cytokine storm induced upon infection[25,26]. Beyond isolated cases of overt severe neurological pathologies, 45% of severe cases exhibited neurologic symptoms[3], increasing the likelihood that potential complications arising from severe SARS-CoV-2 infections may have a strong neurologic component that has yet to be described and characterized. Human coronavirus OC43 has been demonstrated to be transmitted in mice both passive diffusion of released viral particles and axonal transport[27].

Possible pathways for SARS-CoV-2 invasion of the central nervous system

The major receptor for SARS-CoV-2, ACE2, is distributed in multiple tissues of the body. It is present in epithelial cells of alveoli and small intestine arterial and venous endothelial cells and arterial smooth muscle cells[28] neuronal, glial and endothelial cells of the brain[5]. Low expression is observed in glomerular tubular cells. Glomerular endothelial cells, Kupffer cells, hepatocytes, spleen, thymus, lymph nodes and immune cells do not show the expression of ACE2[29]. Similar to SARS-CoV, SARS-CoV-2 uses the spike protein S1 to attach to the host cell ACE2 receptor[13,30], but SARS-CoV-2 binds to the receptor with 10-20 fold higher affinity[30]. A possible explanation for this increased affinity comes from the intense work of Shang *et al.*[31]. By determining the crystal structure of the receptor-binding domain (RBD) of the spike protein (S1), they showed that, compared to SARS-CoV, SARS-CoV-2 receptor binding motif (RBM) contains a structural change in the ACE2-binding ridge caused by a four residue motif (residues 482-485: Gly-Val-Glu-Gly) due to which the ridge becomes more compact and forms more rigid contact with the N-terminal helix of ACE2[31]. In addition, changes in several other residues in the SARS-CoV-2 RBD caused higher stabilization of two virus-binding hotspots at the RBD-ACE2 interface causing higher affinity of SARS-CoV-2 to ACE2[31]. A comparison between SARS-CoV-2 RBD subdomain-1 (S1) with the RBD glycoprotein of bat coronavirus (RaTG13) and S1 protein of SARS coronavirus showed strong but not identical homology in the spike proteins of all three CoVs which can possibly explain the high binding affinity of SARS-CoV-2 to human ACE2 receptor[5]. Ou *et al.*[32] showed that the SARS-CoV-2 spike protein is less thermostable than SARS-CoV spike protein, suggesting its higher infectivity[32].Upon binding with the ACE2, SARS-CoV-2 S protein is primed by serine protease TMPRSS2[33]. SARS-CoV-2 might be able to transmigrate to the brain via the general circulation, the

olfactory pathway, or the peripheral neurons of the lungs. The schematic in Figure 1 shows the possible pathways of virus entry to the central nervous system (CNS).

The olfactory pathway

A proposed pathway that the virus can take to reach the brain is the olfactory pathway [Figure 1B]. Total or partial loss of smell, either by "cytokine storm" or damage of the olfactory epithelium (OE), is an early indicator of SARS-CoV-2 infection. Damage of the OE is particularly important as the cells express both ACE2 and TMPRSS2[34]. ACE2 is highly expressed in the non-neuronal goblet/secretory cells[35] and ciliated receptor cells of the nasal epithelium[5,36]. Upon binding with these receptors, the virus can travel through the cribriform plate to the olfactory bulb, move trans-synaptically to the cortex, and then to the brain stem[5]. The non-neuronal cells in the OE possibly serve as reservoir of the virus[13,34]. The major complication causing death in COVID-19 is respiratory failure and pneumonia. The CNS control of respiration, as described above, is an important factor in maintaining proper respiratory physiology. Failure of CNS to control the rate and depth of respiration may lead to irreversible serious consequences. Brain stem respiratory centers (RC) are the major neuronal groups controlling respiration. In a recent study Moberly *et al.*[37] showed the anatomical and functional connectivity among the RC neurons, nasal epithelium and different brain regions including the olfactory bulb and limbic system[37]. Thus, damage to any segment of this network is expected to cause respiratory distress as well as emotional and behavioral dysfunction. Direct evidence of olfactory transmission of SARS-CoV was produced by Netland *et al.*[13]. By immunohistochemical methods they showed the presence of SARS-CoV in several brain regions of genetically modified mice including olfactory bulb, cerebral cortex, hippocampus and brain stem following intranasal infection of the virus. Most importantly, in case of low-grade infection, the virus was observed in the brain in the absence of lung infection or pneumonia[13]. There is a 60 h delay between virus infection and the presence of virus in the olfactory bulb[13], and this time is probably used by the virus for replication and accumulation in the non-neuronal OE cells[34]. The subsequent transport of the virus to other regions of the brain was relatively faster, approximately 12 h-20 h[13]. However, a recent study conducted by Bao *et al.*[38] showing the respiratory transmission of the virus via liquid droplets from infected to uninfected hACE2 transgenic mice did not report any neural transmission or viral presence in the brain[38].

The respiratory neuronal pathway

Lung-adjacent peripheral neurons may offer a second pathway for SARS-CoV-2 to enter the CNS [Figure 1C]. Due to their proximity to the primary infection site, these neurons are highly susceptible to viral invasion. The previously mentioned porcine HEV has been visually identified by transmission electron microscopy to infect sensory dorsal root ganglia[39] and appears to be trans-synaptically transferred to the brain stem via membrane-coated vesicles[40]. HEV shares 91% homology to hCoV-OC43, a close relative to SARS-CoV-2[41], which provides some evidence that SARS-CoV-2 may also invade via a similar pathway. The direct synaptic connections between these sensory nerves and the respiratory centers in mammals may prove to be important in the characteristic respiratory failure seen in severe COVID-19 patients.

The circulatory pathway

Upon infection, SARS-CoV-2 has been detected in the general circulation from which it can be transported to the cerebral circulation[5]. Endothelial cells (EC) of the blood-brain barrier (BBB) express the ACE2 receptor. The virus then binds with the receptor to enter the ECs and multiply. Eventual budding enables the virus to infect the brain parenchyma, especially the neuronal cells[5,42], which also express ACE2 receptors. The damaged capillaries cause micro-hemorrhage, which can have fatal consequences. Once in the neurons, the viruses multiply and bud off the neurons killing the cells and infecting many more cells in the brain, which leads to functional deficits [Figure 1D]. In the case of SARS-CoV infection, Netland *et al.*[13] showed that the virus causes neuronal damage without evoking a substantial inflammatory response[13]. In a small subset of severe influenza cases and various other viral infections, an associated cytokine storm

Table 1. Currently recruiting clinical trials involving MSCs[1]

NCT number (Phases)	Interventions	Enrollment (age)	Location
NCT04313322 (Phase 1)	Wharton's Jelly MSCs	5 (18+)	Stem Cells Arabia, Amman, Jordan
NCT04336254 (Phase 1,2)	Allogeneic human dental pulp stem cells *vs*. saline injection (Placebo)	20 (18-65)	Renmin Hospital of Wuhan University (East Campus), Wuhan, Hubei, China
NCT04288102 (Phase 2)	MSCs *vs*. 1% Human serum albumin in saline	90 (18-75)	Maternal and Child Hospital of Hubei and Huoshenshan Hospital, Wuhan, Hubei, China
NCT04252118 (Phase 1)	MSCs	20 (18-70)	Beijing 302 Military Hospital of China, Beijing, China
NCT04366271 (Phase 2)	Standard of Care *vs*. MSC	106 (40-80)	Hospital Infantil Universitario Nino Jesus, Madrid, Spain
NCT04355728 (Phase 1/2)	UCMSCs	24 (18+)	University of Miami, USA
NCT04366063 (Phase 2/3)	MSC Therapy	60 (18-65)	Royan Institute, Tehran
NCT04339660 (Phase 1,2)	CMSCs *vs*. Placebo	30 (18-75)	Puren Hospital Affiliated to Wuhan University of Science and Technology, Wuhan, Hubei, China
NCT04392778 (Phase 1/2)	MSCs *vs*. Saline Control	30 (40-60)	Istinye University, Istanbul, Turkey
NCT04390139 (Phase 1/2)	Excel-UMC-Beta *vs*. Placebo	30 (18-75)	Hospital DE Bellvitge, Barcelona, Spain
NCT04371393 (Phase 3)	Remestemcel-L *vs*. Placebo	300 (18+)	University of Southern California, Los Angeles, CA and Ichan School of Medicine at Mount Sanai, NY, USA
NCT03042143 (Phase 1/2)	UCMSCs (CD362 Enriched) *vs*. Placebo	75 (16+)	Belfast Health and Social Care Trust, Royal Hospital, Northern Ireland, UK
NCT04269525 (Phase 2)	Umbilical Cord MSCs	10 (18-75)	Zhongnan Hospital of Wuhan University, Wuhan, Hubei, China
NCT04361942 (Phase 2)	MSC *vs*. Placebo	24 (18+)	Hospital Universitario Rio Hortega, Valladolid, Spain
NCT04333368	Umbilical cord Wharton's Jelly MSCs *vs*. NaCl 0.9%	60 (18+)	Hôpital Pitié-Salpêtrière and Européen Georges Pompidou, Paris, France

[1]ClinicalTrials.Gov; accessed 27 May 2020. MSCs: mesenchymal stem cells

and subsequent BBB breakdown has been described as a known passive method of entry for viral particles causing acute necrotizing encephalopathy in some patients[43].

NEUROTROPISM-TARGETED COVID-19 TREATMENT APPROACH

Thus far, there have been ~300 clinical trials registered in the National Institute of Health (www.clinicaltrials.gov) on treatments for SAR-CoV-2. Most of these trials focus on antiviral drugs, without any due consideration of the neurotropism of the virus. To our knowledge there are more than 160 antiviral agents are available[44]. Screening of these compounds with the intent to identify those that lack neurodegenerative properties or those that possess neuro-regenerative effects may be useful in identifying safe and effective drugs for SARS-CoV-2. It is interesting that one small clinical study relating to SARS-CoV-2 of the effects of mesenchymal stem cells (MSCs), which are known to exert anti-inflammatory and neuro-regenerative activities in brain disorders, showed excellent efficacy, i.e., all 7 of 7 patients showed symptom reduction in 2 days after MSC transplantation[45]. This study has provided a basis for several MSC-based either observational or phase 1-3 trials, which are currently recruiting [Table 1]. In addition, there are several other clinical trials which are registered but have not yet begun to recruit. Given the pivotal role of neuropathology of SARS-CoV-2 and neuronal control of breathing, future therapies should target neurotropism of the virus and combine anti-neuroinflammatory and neuro-regenerative features.

FUTURE THERAPEUTIC IMPLICATIONS

Several lines of evidence on coronaviruses and exploratory studies in SARS-CoV-2 have demonstrated the involvement of viral neurotropism in the brain stem respiratory center, whose components control

breathing, but it is unclear which of these three different pathways offer a potential method of invasion for the virus, and they are not necessarily mutually exclusive. The viral entry of SARS-CoV-2 may be via circulatory in one patient and via oronasal or peripheral sensory neurons in another. Each of these pathways warrants further investigation to determine the neurotropism of SARS-CoV-2, and how these pathways may be exploited to treat patients in the future.

Also, it is evident that one particular approach that is under intense investigation is the development of potential SARS-CoV-2 treatment using MSCs, which have shown neuro-regenerative properties in several brain disorders including traumatic brain injury (TBI)[46]. One of the issues in using MSCs in inflammatory conditions is that MSCs undergo apoptosis or differentiation due to cytokines being released in those inflammatory tissues. It has been reported that pretreatment of TBI animals first with an anti-inflammatory agent followed by MSC treatments significantly increases the efficacy of MSCs[46]. Whether a similar strategy would be more successful in COVID-19 remains to be elucidated. Given the pace and expansiveness of the COVID-19 super pandemic and the fact that the rebound infection may continue for several months to years or could be a seasonal respiratory virus infection similar to influenza and respiratory syncytial virus, it may be prudent to test these possibilities.

DECLARATIONS

Authors' contributions

Wrote the manuscript: Das M, Penn C, Martinez T, Mayilsamy K, McGill A
Made the illustration: Penn C
Reviewed the manuscript: Wiling A, Mohapatra SS, Mohapatra S
Approved the final version of the manuscript: All authors

Availability of data and materials

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Review

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An association between mitochondria and microglia effector function: what do we think we know?

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Abstract

While resident innate immune cells of the central nervous system, the microglia, represent a cell population unique in origin, microenvironment, and longevity, they assume many properties displayed by peripheral macrophages. One prominent shared property is the ability to undergo a metabolic switch towards glycolysis and away from oxidative phosphorylation (OXPHOS) upon activation by the pro-inflammatory stimuli lipopolysaccharide. This shift serves to meet specific cellular demands and allows for cell survival, similar to the Warburg effect demonstrated in cancer cells. In contrast, normal surveillance phenotype or stimulation to a non-proinflammatory phenotype relies primarily on OXPHOS and fatty acid oxidation. Thus, mitochondria appear to function as a pivotal signaling platform linking energy metabolism and macrophage polarization upon activation. These unique shifts in cell bioenergetics in response to different stimuli are essential for proper effector responses at sites of infection, inflammation, or injury. Here, we present a summary of recent developments as to how these dynamics characterized in peripheral macrophages are displayed in microglia. The new insights provided by an increased understanding of metabolic reprogramming in macrophages may allow for translation to the central nervous system and a better understanding of microglia heterogeneity, regulation, and function.

Keywords: Mitochondria bioenergetics, inflammasome, microglia, pro-inflammatory, anti-inflammatory, polarization

INTRODUCTION

The function of innate immune cells such as macrophages is to recognize and respond to a novel stimulus including microbial pathogens and sterile activators. These pro- and anti-inflammatory responses are major sources of soluble molecules, cytokines, hormones, and neuropeptides These factors provide tools to sense, process, and relay physiological signals beyond their canonical roles. Macrophages display a heterogeneous repertoire to fulfill a broad range of functions in host defense, including tissue homeostasis and repair, pathology, and development. To accomplish this, innate immune cells adopt various activation phenotypes. Precise regulation of such activation is essential for maintenance of tissue homeostasis with governance accomplished by a balance of stimulatory and inhibitory signals. Multiple lines of evidence suggest an interlinked relationship between innate immunity and the integrity and function of mitochondria serving to maintain this homeostatic balance. Metabolic pathways provide the necessary energy and serve to regulate phenotype and function. Pro-inflammatory macrophages {M[LPS(+IFNγ)]} display an enhanced glycolytic metabolism and impaired mitochondrial oxidative phosphorylation (OXPHOS). These energy shifts place mitochondria in a pivotal signaling role in macrophage response to stimuli and circumventing immune checkpoint signals[1-3]. The link between immunological and metabolic processes associated with mitochondria, immunometabolism, may influence activation states and polarization of myeloid cells to fine-tune their functions[4-7].

Of the specialized cells of the central nervous system (CNS), basic host defense mechanisms exist predominantly in microglia as resident macrophages. Microglia share many phenotypic characteristics with peripheral macrophages yet are unique in their origin and molecular or transcriptional profile[8-15]. The available literature on the immunometabolism of microglia, as compared to what is known of peripheral macrophages, is limited but growing to address questions of similarities and differences[6,16]. It may also allow for a framework to understand the various other tasks undertaken by microglia during development and chronic maintenance. Here, we present a summary of how these dynamics characterized in peripheral macrophages are displayed in microglia. While much of this work is still somewhat under a "work in progress" classification, even in the peripheral macrophage, new insights provided by an increased understanding of metabolic reprogramming foster a better understanding of macrophage and microglia regulation and function.

MICROGLIA AND IMMUNE CELLS IN THE CNS

The mechanical separation of the CNS from the circulation by the blood-brain barrier[17,18] influences immune responses[19,20] by excluding many peripherally derived innate and adaptive immune cells and inflammatory molecules[21]. However, infiltrating cells significantly contribute to any neuroinflammatory response following disruption of the blood-brain barrier, as can occur with physical injury or high levels of inflammation. In such cases, blood-borne monocytes are allowed to enter the brain parenchyma and, over time, can transition and assume a brain-specific phenotype[22-24]. Additionally, with a T cell-mediated neuroinflammation, peripheral innate immune cells enter the brain as a protective host defense against infection and facilitate repair following stroke or physical trauma[25,26]. In such a case, interactions between microglia and T cells can be signaled via interleukin (IL)-23 and IL-1β, leading to the production of granulocyte-macrophage colony-stimulating factor (GM-CSF) to facilitate microglia proliferation in a manner to promote an appropriate level of response to injury[27]. Recently, the identification of innate lymphoid cells in the brain suggests an additional innate immune cell population that may act to control neuroinflammation[28]. Thus, in such conditions, the macrophage population likely represents a combination of resident microglia and infiltrating monocytes. As a distinction between these two populations, it has been suggested that resident microglia focus on tasks related to maintaining tissue homeostasis while infiltrating cells are involved in severe inflammatory injuries[29,30].

SENSING AND RESPONDING TO THE ENVIRONMENT

While microglia appear to be tightly adapted to the specific requirements within brain regions, they all function in a surveillance mode with mobile processes extending into the surrounding microenvironment to detect tissue changes[31,32]. Upon sensing such changes, microglia respond to their environment via several "sensome" genes, allowing them to sense and interact with their local environment[8,14,33]. These sensome genes include those for putative purinergic receptors, *P2ry12* and *P2ry13,* transmembrane protein 119 (*Tmem119),* G-protein coupled receptor 34 (*Gpr34),* the C-type lectin receptor, the fractalkine receptor, *Cx3cr1,* sialic acid-binding immunoglobulin-type lectin H *(Siglec-h),* and triggering receptor expressed on myeloid cells 2 *(Trem2)*. Siglec proteins contribute to immune regulation by binding sialic acid residues on neurons[34] and TREM2 contributes via recruitment of the immunoreceptor tyrosine-based activation motif-containing adapter protein, DAP-12[35]. The final response of the cell is dictated by the overall pattern of sensome gene activation.

The microglia host-response begins with the recognition of pathogen-associated molecular patterns (PAMPs) such as bacterial, viral, and protozoal products (protein lipid, nucleic acid, and carbohydrate). This occurs via pattern recognition receptors on the plasma membrane or in the endosomal compartments[36], or by binding phagocytic scavenger receptors[37] and macrophage antigen complex I (MAC1, CD11b/CD18), which is a pattern recognition receptor linked to the superoxide-generating enzyme NADPH oxidase[38]. In the absence of microorganisms, a similar but sterile inflammatory response occurs often as a result of trauma, ischemia-reperfusion injury, or chemical exposure[39-41]. Activation in the absence of microbial compounds occurs by endogenous molecules called danger-associated molecular patterns (DAMPs)[42]. Molecules that function as DAMPs include nucleic acids, lipids, and proteins that normally are not present to immune cells until released or unmasked during cell death due to tissue injury. In the CNS, microglia responding in various neurodegenerative diseases in the absence of pathogen have been termed disease-associated microglia (DAM). Intracellular DAMPs include high mobility group box 1 (HMGB1) and peroxiredoxin family proteins. These damage signals can activate immune cells through three major families of intracellular recognition receptors: toll-like receptors, nucleotide-binding domain leucine-rich repeat containing proteins (also known as NOD-like receptors), and Rig1-like receptors. Receptor activation induces specific pathways and the release of cytokines that contribute to injury mitigation[43]. Microglia are influenced by a plethora of factors including receptor agonists[44-48] and transcription factor inducers[49]. It is thought that microglial receptors[50-52] can act as molecular switches to control microglial responses and that many of these actions function through alterations in calcium signals[53]. In all cases, the immediate response upon sensing DAMPs, PAMPs, or other damaging events requires a robust increase in metabolic demand to support actions that initially are beneficial to the homeostatic balance of the nervous system.

Injury-induced inflammatory processes are dynamic and demonstrate spatial and temporal heterogeneity[54-56]. In general, characterization of the macrophage response is based on the nature of the activating stimulus and the resulting production of factors[57]. A conceptual framework has been proposed that suggests the nature of the activating stimulus can drive a range of activation phenotypes[58-62], and it has been used as a basis for characterizing cellular responses[63-70]. While phenotypic activation-state distinctions are currently under scrutiny[67,71], it has been shown that classically activated microglia associated with inflammation can be produced upon stimulation with agonists for toll-like receptors (e.g., lipopolysaccharide, LPS) or IFNγ receptors. In contrast, different aspects of the immune response that do not involve the classical response can be observed upon stimulation by IL-4 or IL-13 with the expression of anti-inflammatory cytokines (IL-4, IL-10, IL-13, and TGF-β), arginase-1 (Arg1), CD206, and Chitinase-3-like-3 (Ym1 in rodents)[72-76]. It is considered that the different phenotypes may be related, yet have different roles in host defense, wound healing, and resolution of inflammation[57,60]. Differences in metabolic processes have been identified across these different activation inducers, suggesting a role for mitochondria in phenotypic outcome[77].

STIMULUS-DRIVEN METABOLIC RE-PROGRAMMING OF MICROGLIA

In a normal "resting" cell, energy demands are addressed with the conversion of glucose to pyruvate with entry into glycolysis. Pyruvate in the cytosol can be taken up by mitochondria and enters the tricarboxylic acid (TCA) cycle where it is oxidized to generate ATP. This provides a total energy gain of approximately 36 ATP per one molecule of glucose. In contrast, with hypoxia or anoxia, the cell has the ability to divert pyruvate away from mitochondria OXPHOS, allowing for ATP generation during low oxygen conditions. In this case, one glucose molecule will generate two pyruvate molecules that will be converted to lactate by lactate dehydrogenase in the cytosol[78,79]. While this reaction generates significantly fewer molecules of ATP, glycolysis proceeds due to the production of NAD^+. While less efficient, a beneficial effect of a shift to glycolysis is that it can be very quickly induced to meet cellular demands in cells with high glucose capacity[80]. The importance of this shift was initially demonstrated in cancer cells in what is known as the Warburg effect[81,82]. In cancer, malignant cells shift their demand for biosynthetic precursors and energy change and change their metabolic profile from a relatively low rate of glycolysis and the oxidation of pyruvate by the TCA cycle. The shift in metabolic profile is characterized by a lower rate of OXPHOS, high rate of glycolysis, and elevated lactic acid production. The high glycolytic rate induced during the Warburg effect is driven by the need to meet the increased demand for production of nucleotides and amino acids. While this effect was initially identified and characterized in cancer cells, a similar ability to utilize such a metabolic switch has been demonstrated in immune cells to meet increased energy demands when responding to infection or injury[1].

There is now evidence suggesting a role for metabolic reprogramming by mitochondria in the maintenance and establishment of innate and adaptive immune responses[75,83-96]. Given that immune cell populations depend on unique effector functions in response to distinct stimuli that often require production and secretion of high amounts of signaling factors and antimicrobial agents, it follows that changes in mitochondria function to meet these demands are crucial for efficient response to distinct contexts[97-101]. It was initially observed that, upon activation, macrophages increase glycolysis and decrease oxygen consumption[102,103]. It was further demonstrated that macrophage phenotype can be shifted by reprogramming glucose metabolism[104,105], which helps meet energy demands required for shifting cell function and survival[106].

Under normal conditions, microglia exist in a surveillance phenotype for constant monitoring of the parenchyma[107,108] and preferentially rely on oxidative metabolism[90,109,110]. Upon activation by LPS[94], amyloid-b[111], and iron loading[112,113], microglia switch their reliance on OXPHOS metabolism[69,110,114,115] towards glycolytic metabolism to maintain mitochondrial function and ensure cell survival[91,94,95,116]. Voloboueva *et al.*[94] showed that, upon stimulation by LPS, BV-2 microglia increased lactate production and decreased mitochondria oxygen consumption and ATP production. This shift was reported to be modulated by mitochondrial glucose-regulated protein 75/mortalin[94]. Exposure to a combination of LPS and IFN-γ increases nitric oxide formation, glucose consumption, hexokinase activity, glucose-6-phosphate dehydrogenase activity, phosphofructokinase-1 activity, lactate dehydrogenase activity, and lactate release, suggesting potentiated glycolysis[94]. Similar findings were reported by Orihuela *et al.*[69]: following LPS, BV2 microglia and primary murine microglia shifted from a primary oxidative metabolic towards glycolytic metabolism with no evidence of cell death. An increase in microglial mitochondria has been observed with activation[117,118], implicating an association with mitochondria biogenesis. Recent studies have suggested that a shift in glycolysis in microglia is accompanied by an increase in the enzyme PFKFB3, which is responsible for activation of phosphofructokinase[119]. Additionally, this metabolic shift has been found to be regulated by the anti-inflammatory cytokine IL-10 for aerobic glycolysis inhibition and OXPHOS[120].

In a non-classical activation state, macrophages use oxidative metabolism for functions involved in normal maintenance functions, tissue repair, and wound healing[73,121,122]. In IL-4 stimulated macrophages [M(IL-4)],

the Krebs cycle and OXPHOS remain intact. In addition, the cells are able to utilize fatty acid oxidation and oxidative respiration for energy production[98,123-125] and arginine metabolism is shifted to ornithine and polyamines[126]. Work by Ferger *et al.*[118] suggested that the stability of the electron transport chain in mitochondria plays a more substantial and critical role for the microglia response to IL-4 as compared to the response to LPS. In microglia, exposure to LPS induced a rapid and transitory decrease in the mitochondrial uncoupling protein-2 (UCP-2) levels accompanied by increased mitochondrial reactive oxygen species (mtROS) production. In UCP-2-silenced microglia, the response to LPS was exacerbated and a response to IL-4 was eliminated[127]. An earlier study examining the translation of responses in macrophages to microglia reported reduced glucose consumption and lactate production in BV-2 cells exposed to IL-4[109]. It was suggested that this phenotype was associated with phagocytosis of debris and the reduced need for anabolic reactions. Similar findings were reported by Orihuela *et al.*[69] with exposure of BV-2 cells or primary murine microglia to IL-4/IL-13 in that the cells remained within an oxidative metabolic state with OCR and ECAR levels similar to non-stimulated cells. There were also elevations in mRNA levels for *Ym1, Il4, Cd163*, and *Arg1*, but no induction of *Tnfa* or *Il1*[69]. The lack of a demonstrated metabolic shift with IL-4/IL-13 stimulation is in contrast to observations in peripheral macrophages of stimulated glucose uptake in addition to fatty acid metabolism and shift in mitochondrial biogenesis[125].

MITOCHONDRIA AND FREE RADICAL PRODUCTION

A key feature of classically activated macrophages is their ability to produce reactive oxygen species (ROS) to facilitate killing of phagocytized bacteria[128]. Stimulation of macrophages with LPS and IFN-γ increases inducible nitric oxide synthase (iNOS), generating nitric oxide (NO), a reactive nitrogen species that can inhibit mitochondrial respiration by nitrosylating iron-sulfur proteins in electron transport chain complexes and cytochrome c oxidase[129,130]. It is considered that iNOS and NO-mediated inhibition of mitochondrial metabolism in macrophages is essential for the metabolic switch activated by LPS. This is not as well established in microglia, especially given that, while nitric oxide production is often linked with pro-inflammatory cytokines, such cytokines can be stimulated by sterile activators in the absence of NO. In microglia, it has been proposed that activation of the rapamycin (mTOR) pathway may actively contribute to this process[131] as well as pro-inflammatory cytokine production and phagocytic activity[132-134]. The resulting elevated thiamin pyrophosphate activity increased production of purines and pyrimidines, which yield nicotinamide adenine dinucleotide phosphate (NADPH) for the NADPH oxidase enzyme and ROS production[135] implicated in the transition of microglia to a pro-inflammatory phenotype[136-139]. It has been proposed that glycolytic ATP production may utilize the electron transport chain to compensate for this shift towards ROS production[128]. It is known that superoxide produced by NADPH oxidase is predominantly extracellular. *In vivo*, extracellular superoxide dismutase 3 (SOD3) forms membrane permeable H_2O_2. Studies have suggested that H_2O_2, rather than SOD, serves as the primary ROS involved in mediating microglial activation and proliferation in response to pro-inflammatory stimuli[136,140,141]. H_2O_2 has also been implicated in the increase in CD11b expression both *in vitro* and *in vivo*[142], as well as in persistent neuroinflammation related to impaired NF-kB p50 function[143]. Superoxide anion is the primary ROS produced by mitochondria and mitochondria-derived H_2O_2 and, in addition to NADPH oxidase, may contribute to a pro-inflammatory phenotype of microglia such as that observed with the mitochondrial toxin, rotenone[144]. With a response sufficient to result in ROS production, the associated intracellular damage is limited by increased generation of NADPH required for maintenance of reduced glutathione and nitric oxide production[145,146].

GLUCOSE

In addition to the critical role that glucose plays in energy metabolism, it serves as an exclusive substrate for the hexose monophosphate shunt, which produces NADPH that is required by glutathione reductase to convert oxidized glutathione (GSSG) back to reduced glutathione (GSH). It also serves to quench ROS and

repair oxidative damage through glutathione- and thioredoxin-coupled pathways[147]. Glucose metabolism influences microglial activation through an NADH-sensitive co-repressor termed C-terminal binding protein (CtBP). Slowed glucose flux through glycolysis reduces NADH levels and reduce NADH:NAD^+ ratio[148]. In both microglia and macrophage RAW264.7 cells, glucose flux regulates iNOS expression and other pro-inflammatory genes through effects on cytosolic NADH:NAD^+ ratio and CtBP[149].

Several glucose transporters such as GLUT1[150], GLUT3[151], and GLUT5[152] are expressed in microglia. Acute fluctuation of available glucose impacts microglia activity with an elevated response to LPS upon shifting from a normal to high glucose level. Shifting from a high to normal glucose level can also induce metabolic stress[153]. Glucose levels can influence pro-inflammatory gene transcription by several mechanisms. One such mechanism relies on the formation of advanced glycation end-products (AGE). These products consist of modified proteins and lipids as a result of non-enzymatic reactions with sugars. It is known that microglia express receptors of AGE and, upon activation, pro-inflammatory signaling pathways are stimulated[127,154]. In peripheral macrophages, it has been reported that a shift in the cell's energy source induced by glucose deprivation results in an altered response to a pro-inflammatory stimulus[155-157]. Multiple studies have reported an inability of microglia to respond appropriately to LPS under oxygen and glucose deprivation or with 2-DG inhibition of glucose metabolism[109]. However, there is evidence that microglia are capable of functioning with alternative energy sources to adequately respond to an inflammatory challenge. Choi *et al.*[158] reported an increase in mRNA and protein levels for IL-6 in microglia after 7 h of glucose and serum free medium. Upon stimulation with LPS, glucose-deprived microglia retained their normal ability to respond with elevations in nitrite, IL-1β, and TNFα[159]. Primary rat microglia shifted to glucose-free medium for 1 h to LPS showed an exacerbated release of NO within 24 h and similar elevations in TNFα and IL-1β as compared to non-glucose-deprived cells. Glucose deprivation for 24 h prior to LPS exposure increased release of IL-1β with no deficits in NO or TNFα. The authors suggested that microglia were able to mobilize fatty acids from intracellular lipid droplets as an energy source. The majority of studies examining the effects of glucose deprivation have focused on relatively short-term exposures, within 1-24 h. While these studies demonstrated that both peripheral macrophages and microglia can shift their response to a pro-inflammatory stimulus in a selective manner, the question remains as to whether such a response would be altered when the cells were forced to a more prolonged shift in energy metabolism. When RAW 264.7 [Figure 1] or BV-2 [Figure 2] cells were maintained for three days under culture conditions to force cells to rely on galactose as an alternative energy source, the cells were able to normally respond to LPS stimulation. However, the diminished pro-inflammatory cytokine response observed when 2-DG was used in previous studies to inhibit glycolysis may have been related to the lower basal OXPHOS induced[160]. The differences across these studies likely lie with the method of depleting glucose: removing glucose from the medium; the addition of 2-DG, which in and of itself can lower basal induction of OXPHOS[160]; or the combination of glucose deprivation with hypoxia. In RAW cells, the morphological changes observed with LPS activation have been demonstrated to be diminished under galactose, suggesting a requirement of glucose to facilitate cell spreading[161]. This was not clearly observed in the current study where similar LPS-induced morphological patterns were observed in the absence of pyruvate [Figure 1]. In BV-2 cells, a slight morphological shift was observed with the low level of LPS stimulation with minimal induction of nitrate and an elevation in TNFα and IL-1 protein.

GLUTAMINE

Macrophages utilize glutamine at high rates to synthesize amino acids, nucleotides, NADPH, and energy production and are dependent upon extracellular sources of the amino acid[103]. Channeling of glutamine into the Krebs cycle is a primary route to promote succinate synthesis in macrophages. This occurs with glutamine being used for synthesis of glutamate, GABA, and succinate, bypassing the TCA cycle[155]. This stabilizes hypoxia-inducible factor 1-alpha (HIF-1α), an oxygen-sensitive transcription factor that allows the

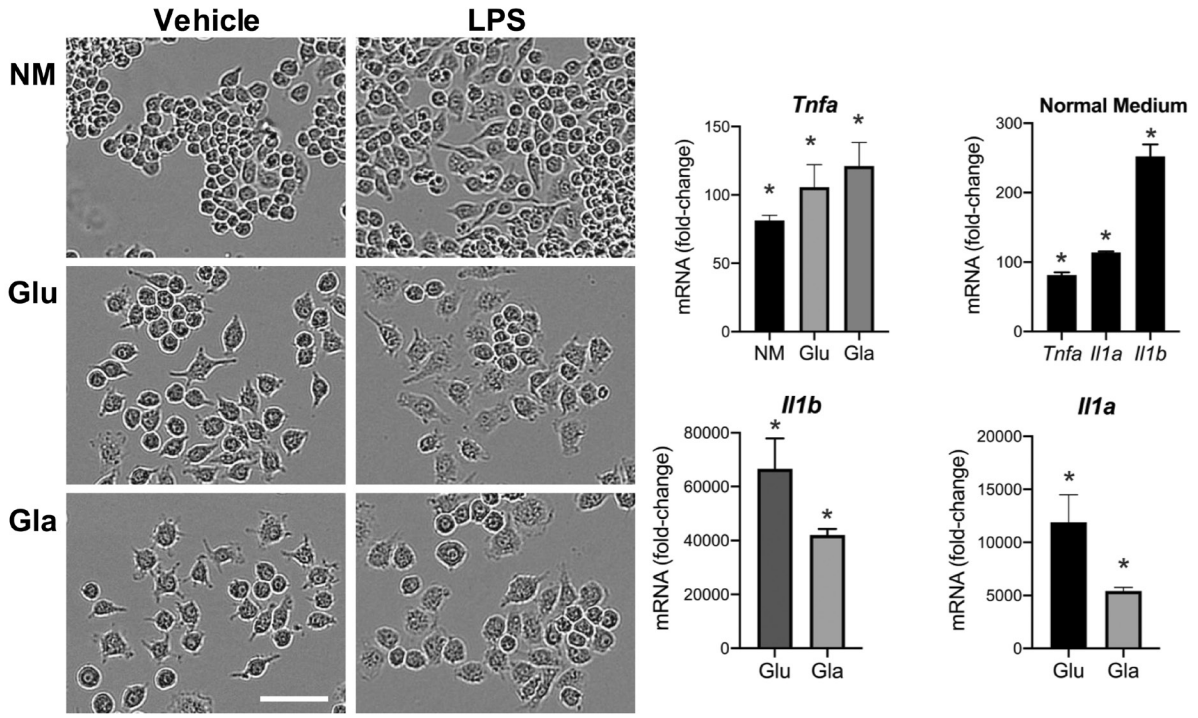

Figure 1. Response of RAW 264.7 cells to LPS under glucose or galactose medium conditions. RAW 264.7 cells were plated in 6-well tissue culture plates (Corning, Corning, NY) and maintained in normal growth medium (NM) [DMEM (Gibco, ThermoFisher, Waltham, MA) containing 4.5 g/L glucose, 2 mM L-glutamine, sodium pyruvate, supplemented with 100 U/mL penicllin/ streptomycin (Sigma-Aldrich, Burlington, MA) and 10% fetal bovine serum (FBS #1 00-106, 0.25 endotoxin units/mL; Gemini Bio-Products, Sacramento, CA) and allowed to reach 85% confluence over 3-5 days. Cells were maintained at 37 °C, 5% CO_2/5% O_2, 90% humidity (Nu-5831 tri-gas incubator, Nuaire, Plymouth, MN). NM was changed to phenol-free NM medium or phenol-free DMEM medium containing 2 mM L-glutamine and 100 U/mL penicillin/streptomycin supplemented with (2) high glucose (25 mM; Glu), or 3] galactose (10 mM; Gla). Cells were maintained in the experimental medium for 3 days following which, under the same media conditions, cells were exposed to lipopolysaccharide (LPS; 1 μg/mL; Sigma) for up to 18 h and monitored using a live cell imaging system (IncuCyte, Sartorius) under normal incubator conditions. (A) Representative images (20x) of cell morphology under normal medium (NM), high glucose (Glu), or galactose (Gla). Scale bar = 25 microns. (B) Samples were collected at 3 h post-LPS and mRNA isolated using TRIzol® Reagent (Invitrogen, Carlsbad, CA), and 2.5 μL cDNA was used for qRT-PCR for *Tnfa*, *Il 1a*, *Il 1b*, and *cyclophilin A* using TaqMan™. Individual gene expression levels were normalized to cyclophilin A and presented as fold-change from vehicle controls in each medium condition. Data were analyzed by 2-way ANOVA followed by Dunnett's test for independent group mean comparisons. Data represent mean +/- SEM (n = 3-4). *Significance level as compared to vehicle control set at $P < 0.05$

cell to adapt to a hypoxic environment[162]. It has been suggested that HIFs function to facilitate cross-talk between inflammation and metabolism[163]. HIF-1a can induce the expression of pro-inflammatory cytokines and has been proposed to serve a role in shifting glycolytic pathways to favor anaerobic metabolism[114]. With classic activation, succinate regulates HIF-1a to drive a sustained production of IL-1β[100] and the subsequent cell actions serve to maintain the macrophage survival. For the non-classical phenotype, glutamine metabolism acts at multiple levels including the generation of a-ketoglutarate and serves as a substrate for UDP-GlcNAc synthesis[164].

NLRP3 INFLAMMASOME

One biological response to an inflammatory event that is critically dependent upon metabolic regulation is inflammasome activation. This is especially relevant for inflammasomes that require prior cell priming for full activation upon a secondary stimulus. For example, glycolytic rate can influence formation of NLRP1 and NLRP3 inflammasomes in macrophages[165,166]. Inflammasomes are multiprotein complexes formed in the cytosol of immune and neural cells in response to pathogenic and danger signals. They consist of a cytosolic sensor belonging to the AIM2 (absent in melanoma 2), or NLR, an adaptor protein

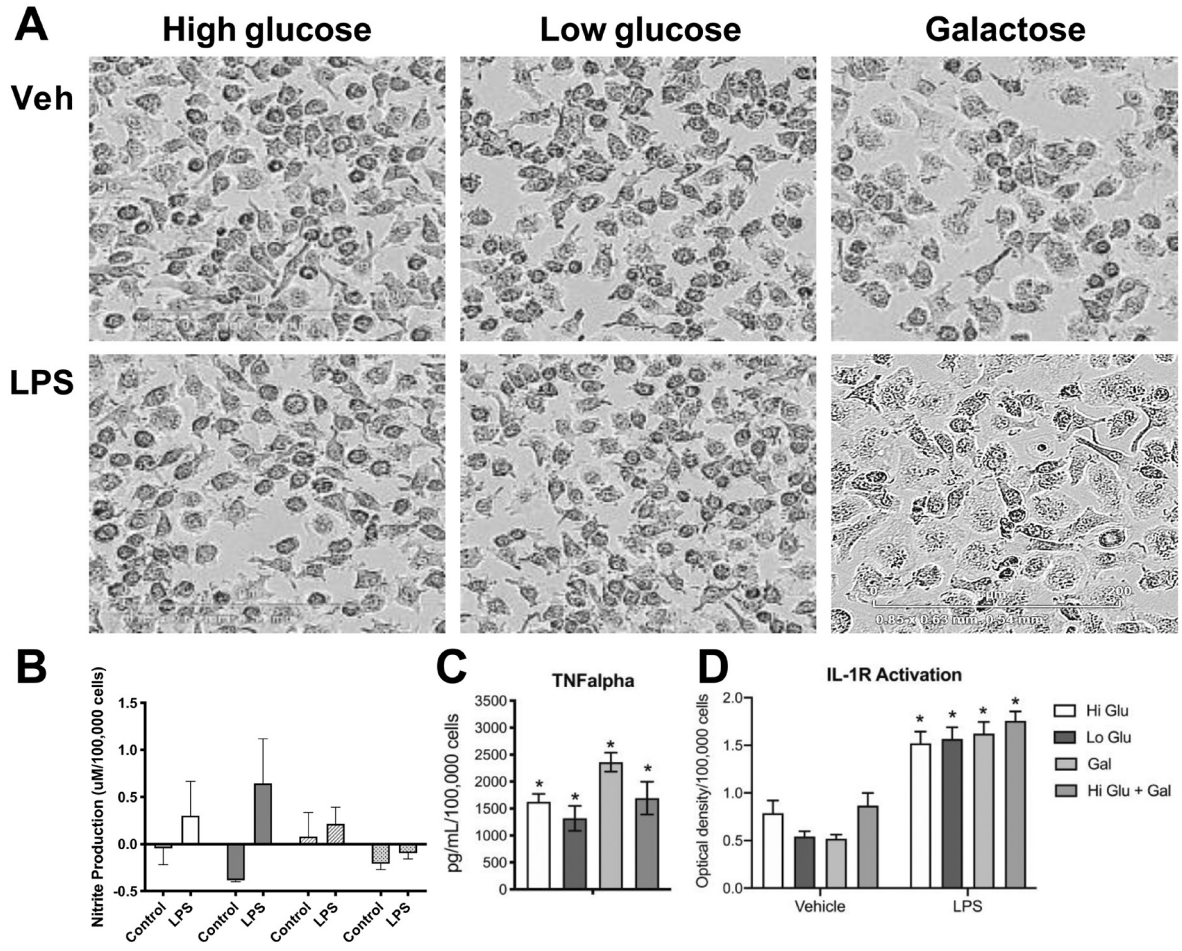

Figure 2. Response of BV-2 cells to LPS under glucose or galactose medium conditions. BV-2 murine microglia cells were plated in 6-well tissue culture plates (Corning, Corning, NY) and maintained in normal growth medium (DMEM (Gibco, ThermoFisher, Waltham, MA) containing 4.5 g/L glucose, 2mM L-glutamine, supplemented with 100 U/mL penicillin/streptomycin (Sigma- Aldrich, Burlington, MA) and 10% fetal bovine serum (FBS #100-106, 0.25 endotoxin units/mL; Gemini Bio-Products, Sacramento, CA) and allowed to reach 85% confluence over 3-5 days. Cells were maintained at 37 °C, 5% CO_2/5% O_2, 90% humidity (Nu-5831 tri gas incubator, Nuaire, Plymouth, MN). The normal growth medium was changed to phenol-free complemented DMEM medium containing 2mM L-glutamine, supplemented with 100 U/mL penicllin/ strepto supplemented with 1) high glucose (25 mM), 2) low glucose (10 mM), 3) galactose (10mM), or 4) high glucose + galactose). Cells were maintained in the experimental medium for 3 days following which, under the same media conditions, cells were exposed to lipopolysaccharide (LPS; 100 ng/mL; Sigma) monitorec using a live cell imaging system (IncuCyte) under normal incubator conditions for up to 18 h. A: Representative images (20x) of cell morphology under high glucose (Hi Glu), low glucose (Lo Glu), or galactose (Gal). Scale bar = 200 microns; B: Nitrite accumulation in culture medium was measured as an indirect indicator of nitric oxide synthesis using a Greiss Reagent kit (Promega, Madison, WI) following manufacturer s instructions. Estimates were determined relative to standard curve; C: Estimates of the release of IL-1 into the medium were obtained using HEX Blue™ IL-1R cells (InvivoGen, San Diego, CA) following manufacturer's instructions. From 20 μL aliquot of the medium, after a 1.5 h incubation at 37 °C, absorbance at 620 nm was measured using a BioTeck Synergy 4 plate reader. Data were calculated relative to background control; D: TNFα protein levels were determined by Mouse TNFα ELISA MAX kit (BioL egend, San Diego, CA) with BD OptEIA Reagent Set B according to manufacturer' instructions. Absorbance was measured at 450nm with a 570nm background subtraction. Protein levels were determined based on standard curve and calculated relative to total cell number as determined using IncuCyte software. Data represent mean +/- SEM (n = 5-6). Data were analyzed by 2-way ANOVA followed by Bonferroni post-hoc comparisons. *Significance level as compared to vehicle control set at $P < 0.05$

ASC (apoptosis-associated speck-like protein containing a CARD), and an effector caspase, primarily caspase-1. There are a variety of inflammasomes, most of which fall into the NLR domain[167]. NLRP1 and AIM2 inflammasomes have been characterized in neurons[168-170] and the NLRC4, NLRP2, and NLRP3 inflammasomes in astrocytes[171,172]. Components for multiple inflammasomes are expressed in microglia[173,174]. The NLRP3 inflammasome responds to a number of activators, including sterile activators,

such as asbestos, silica crystals, aluminum salts, and polystyrene nanoparticles[175-178], and aberrant proteins, such as extracellular Aβ[179], thereby contributing to a broad range of common inflammatory pathologies and chronic inflammation.

The NLRP3 inflammasome responds to metabolic regulation[180] and has been increasingly recognized as a bridge between mitochondrial damage sensing and pro-inflammatory signaling within monocytes, including microglia[181,182]. Unlike most inflammasomes, NLRP3 typically requires a two-step activation and it is this process for which there is mounting evidence that mitochondrial damage plays a contributing role[101]. Activation of TLR, tumor necrosis factor receptor, or interleukin-1 receptor (IL-1R) initiates an intracellular cascade of effects, including activation of NF-kB. This upregulates NLRP3 and pro-IL-1β within the cell and facilitates post-transcriptional changes to NLRP3 to free ubiquitinated binding sites by BRCC3 (BRCA1/2-containing complex subunit 3)[176,183,184]. Delivery of a secondary "trigger" such as PAMPs, DAMPs, or intact pathogens to the "primed" cell causes the release of the repressed state of NLRP3. Upon release, NLRP3 activates the inflammasome forming a multiprotein complex comprised of the cytosolic sensor NLRP3, ASC, and caspase 1[185]. Caspase 1 facilitates the cleavage of the pro-forms of IL-1β and IL-18[186], resulting in the release of mature protein[187]. The release of active IL-1 family cytokines is normally related to pyroptotic cell death; however, in the absence of cell death, hyperactivity of cells and the recruitment of a process dependent on plasma membrane-localized pores can result in similar protein release[188,189]. While inflammasome activation is an efficient producer of mature IL-1β, inflammasome independent mechanisms exist, including cathepsin B or caspase 11 dependent pathways[190,191], bacterial pore-forming toxins, and extracellular ATP[177]. Thus, an upregulation of mature IL-1β does not automatically indicate an inflammasome mechanism. In addition to the release of inflammatory factors, the physical release of ASC specks into the extracellular environment represents a stimulus for activating phagocytic cells in the immediate environment, thus contributing to a prolonged propagation of inflammation[192] or other biological responses[193].

Induction of mitophagy, the process by which cells clear damaged mitochondria, has been implicated in inhibition of NLRP3 signaling[194]. Release of oxidized mitochondrial DNA (mtDNA) produced during the priming stage[195,196] can interact with the NLRP3 receptor and induce inflammasome activation. Nakahira *et al*.[196] reported that inhibition of mitophagy in macrophages heightened the NLRP3 inflammasome activation in parallel with uncleared mitochondrial DNA released into the cytosol. The mitochondrial cytidine/uridine monophosphate kinase-2 (CMPK2) is a nucleotide kinase required for mtDNA synthesis and production of oxidized mtDNA fragments. These fragments can act as activating ligands for the NLRP3 inflammasome complex[197]. In addition, the release of mtROS triggered by small molecule inhibition of complex I and III has been associated with NLRP3 inflammasome activation[198]. The association between mtROS as a trigger for NLRP3 inflammasome activation remains controversial given potential off-target effects of mtROS inhibitors. While studies have reported a role for mtROS in NLRP3 inflammasome activation, other conflicting studies have been reported. At least one study reported that mtROS inhibitors do not block the secondary activation step, but rather the initial priming step[199]. Apart from acting as an activator of NLRP3, mitochondria can act as a docking system for inflammasome assembly. This interaction is driven by the externalization of mitochondrial lipid cardiolipin from the inner membrane to the outer membrane, which then independently interacts with caspase-1 and NLRP3[200].

NLRP3 inflammasome activation in microglia has gained attention as a contributing mechanism in several neuroinflammatory disease pathologies including Alzheimer's disease, amyotrophic lateral sclerosis, multiple sclerosis, and Parkinson's disease[201-205]. While much is similar between the biochemistries of microglia and macrophages, differences in inflammasome activation have been reported. For example, exposure of microglia cells to the antioxidant NAC did not affect LPS priming yet inhibited Aβ 1-42 peptide stimulation of caspase-1 dependent IL-1β secretion[202]. While microglia show similar expression

of inflammasome components and response to stimulus, the dependency on caspase-1 for IL-1β secretion is only partial and a higher level of mature IL-1β secretion is observed with longer periods of priming than in hematopoietic macrophages. Prolonged IL-1β secretion from microglia likely occurs as a result of a deficit in negative regulation mechanisms as compared to macrophages. As an additional consideration, activation of the inflammasome in peripheral macrophages serves in a regulatory capacity in the induction of pyroptotic cell death to remove the damaging immune cell. How this translates to microglia remains in question given the long-lived nature of these cells.

CONCLUSION

The interest in metabolic functions of microglia has evolved from our knowledge of mitochondrial demands and responses of peripheral macrophages in their various effector functions. Recent findings have set the framework for an association between the metabolic status of immune cells with the characteristics of an immune response against pathogens. The majority of studies have relied on different pro-inflammatory stimuli, such as virus, GM-CSF, LPS, LPS + INFγ, or IL-4, to induce cells to examine macrophage metabolism *in vitro*. However, the resulting phenotype and metabolic profiles can differ with not all stimuli leading towards glycolysis. Conversely, the diverse non-inflammatory stimuli normally examined, i.e., IL-10, glucocorticoids, IL-13, M-CSF, and IL-4, are grouped together[74]; however, similar to the pro-inflammatory stimuli, the phenotypic change may differ. This is not unexpected given that macrophage activation states display multiple profiles depending on the initiating stimuli[206]. However, examination of metabolic adaptations of macrophages has demonstrated that such adaptations are critical factors regulating a variety of immune cell responses. The need to rapidly modulate cellular responses to pathogen or inflammatory signals demands a remodeling of the metabolic pathways to execute such actions. While many of the basic responses translate across peripheral macrophages and microglia, the uniqueness of microglia suggests that this may not be a complete translation across cells. Additionally, the limited range of inducing stimuli examined in microglia cells raises the question of how the cells will compare given a broader range of stimuli. Further exploration of similarities and uniqueness will contribute to our understanding of the interplay between metabolism and immune cell responses as they apply to the nervous system. It may also offer a framework from which to address issues of translation between experimental animal data to human disease conditions that involve the innate immune system[207-209]. Understanding the mitochondrial-related characteristics of microglia will likely be critical in identifying successful therapeutic approaches to the detrimental effects of neuroinflammation or in facilitating repair.

DECLARATIONS

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Authors' contributions

Contributed to the conceptualization, design and interpretation of the experiments and in manuscript preparation: Childers G
Generated the original data: Lopez Hernandes I, Giridharan S
Contributed to the conceptualization of the manuscript, data interpretation, and writing of the final manuscript: Harry GJ

Availability of data and materials

Not applicable.

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

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Phf15 - a novel transcriptional repressor regulating inflammation in a mouse microglial cell line

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Abstract

Aim: Excessive microglial inflammation has emerged as a key player in mediating the effects of aging and neurodegeneration on brain dysfunction. Thus, there is great interest in discovering transcriptional repressors that can control this process. We aimed to examine whether *Phf15* - one of the top differentially expressed genes in microglia during aging in humans - could regulate transcription of proinflammatory mediators in microglia.

Methods: Real-time quantitative PCR was used to assess *Phf15* mRNA expression in mouse brain during aging. Loss-of-function [short hairpin RNA (shRNA) -mediated knockdown (KD) and CRISPR/Cas9-mediated knockout (KO) of *Phf15*] and gain-of-function [retroviral overexpression (OE) of murine *Phf15* cDNA] studies in a murine microglial cell line (SIM-A9) followed by immune activation with lipopolysaccharide were used to determine the effect of *Phf15* on proinflammatory factor (*Tnfα*, *IL-1β*, and *Nos2*) mRNA expression. RNA sequencing was used to determine global transcriptional changes after *Phf15* knockout under basal conditions and after lipopolysaccharide stimulation.

Results: *Phf15* expression increases in mouse brain during aging, similar to humans. KD, KO, and OE studies determined that *Phf15* represses mRNA expression levels of proinflammatory mediators such as *Tnfα*, *IL-1β*, and *Nos2*. Global transcriptional changes after *Phf15* KO showed that *Phf15* specifically represses genes related to the antiviral (type I interferon) response and cytokine production in microglia.

Conclusion: We provide the first evidence that *Phf15* is an important transcriptional repressor of microglial inflammation, regulating the antiviral response and proinflammatory cytokine production. Importantly, *Phf15* regulates both basal and signal-dependent activation and controls the magnitude and duration of the microglial inflammatory response.

Keywords: *Phf15*, microglia, transcriptional repression, neuroinflammation

INTRODUCTION

Microglia are the resident myeloid-lineage cells of the brain. They actively provide homeostatic surveillance of the brain parenchyma, playing critical roles during development, maintenance, and repair throughout the life of an organism. As innate immune cells, however, microglia are also capable of mounting a full inflammatory response to environmental challenge in order to clear threats and restore homeostasis[1-6]. Microglia express pattern recognition receptors including Toll-like receptors to sense changes in their environment, such as infection by pathogens or endogenous danger signals. They can then respond by releasing proinflammatory mediators such as tumor necrosis factor alpha (TNFα), interleukin 1 beta (IL-1β), IL-6, reactive oxygen species, and reactive nitrogen species including nitric oxide (NO) to protect against threats[1,5,7].

Although beneficial when their production is tightly controlled, deregulated or sustained microglial production of inflammatory mediators can lead to collateral damage of surrounding neurons and other cells[5,7,8]. Thus, the transition to an activated state, as well as timely resolution of the inflammatory response, must be tightly regulated. Increasing evidence suggests that, during aging, microglia lose homeostatic function and acquire a proinflammatory phenotype that exacerbates aging-related brain dysfunction[9]. Indeed, aberrant microglia activation has been found in many types of age-related neurodegenerative conditions, for example Parkinson's disease (PD) and Alzheimer's disease (AD), which are marked by inflammatory processes involving glia, and microglia in particular[9-11].

Since excessive production of proinflammatory mediators is neurotoxic[8,12-14], various molecular mechanisms exist to regulate transcriptional repression of inflammatory gene expression. For example, basal state repression, that is, before the arrival of an activating signal, is generally carried out via recruitment of co-repressor complexes that prevent initiation of inflammatory gene transcription. After stimulation by an activating signal, additional mechanisms can maintain quiescence by restraining active transcription. Finally, numerous mechanisms mediate the timely resolution of the inflammatory response at the transcriptional level, including transrepression mechanisms that can remove transcription factors from inflammatory gene promoters[8,15-18].

Studies have also highlighted an important role for chromatin modifications in the transcriptional control of inflammatory gene expression[19,20]. A recent study by Soreq *et al.*[21], which compared transcriptional profiles of different brain cell types and regions throughout healthy human aging, found microglial gene expression profiles as being one of the most predictive markers of biological age in the brain. The same study identified a relatively unknown gene, PHD finger protein 15 (*PHF15*), among the top 25 differentially expressed genes in microglia during aging. Work in embryonic stem cells, as well as sequence and structural similarity to other members of the PHF family, indicate that PHF15 is a putative chromatin-mediated gene regulator[22].

Given that aging skews microglia towards a proinflammatory phenotype, and that *PHF15* was found to be highly upregulated during non-pathological aging, we sought to determine whether *Phf15* might regulate microglial inflammatory function. We found that *Phf15* strongly represses proinflammatory gene expression, regulating both basal and signal-dependent activation and modulating the magnitude and duration of the mouse microglial inflammatory response. Importantly, *Phf15* seems to regulate proinflammatory and interferon type I (IFN-I)-dependent gene expression. Increased IFN-I tone and proinflammatory cytokine expression are both hallmarks of the aging brain[23-26]. Our findings suggest that *Phf15* is an important novel repressor of microglial inflammatory function that might work to counteract age-induced inflammation in the healthy, aging brain.

METHODS

Animals

Adult male C57Bl6/J mice were purchased from The Jackson Laboratory and maintained on a 12-h/12-h light/dark cycle (lights on at 07:00) with *ad libitum* access to food and water and aged for ~2.5, ~14, or ~20 months. All animal care and procedures were approved by the University of California, Berkeley Animal Care and Use Committee.

shRNA-mediated knockdown of *Phf15* in murine microglial cells

pGIPZ lentiviral mouse *Phf15* shRNA constructs or a control scrambled shRNA were purchased from Dharmacon (Lafayette, CO). Lentivirus was packaged via co-transfection of each pGIPZ shRNA with pCMV-VSV-G (Addgene plasmid #8454)[27] and pCMV-dR8.2 (Addgene plasmid #8455)[27] into HEK 293T cells using Lipofectamine 3000 reagent (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. Viral supernatant was harvested after 48 h and incubated with SIM-A9 murine microglial cells in SIM-A9 complete medium [DMEM/F12 (Life Technologies, Carlsbad, CA), 10% fetal bovine serum (FBS; GE Healthcare Life Sciences, Chicago, IL), 5% horse serum (HS; GE Healthcare Life Sciences, Chicago, IL), and 1% Penicillin-Streptomycin (Life Technologies, Carlsbad, CA)]. After 48 h, GFP+ cells were sorted by Fluorescence-activated cell sorting (FACS) on an Aria Fusion (BD Biosciences, San Jose, CA; UC Berkeley Cancer Research Laboratory), expanded, and subcultured for immune stimulation experiments. Percent knockdown (KD) was determined via real-time quantitative PCR (RT-qPCR).

Overexpression of *Phf15* in murine microglial cells

A *Phf15* overexpression (OE) vector was constructed by cloning the full length *Phf15* cDNA (*Mus musculus* PHD finger protein 15, mRNA cDNA clone MGC:143877 IMAGE:40094330) obtained from Dharmacon (Lafayette, CO) into a pMYs-IRES-GFP retroviral vector (Cell Biolabs Inc., San Diego, CA). Viruses expressing the full length *Phf15* cDNA or empty vector control were co-transfected with pCL-10 A1 (Addgene plasmid #15805)[28] in HEK 293T cells using Lipofectamine 3000 (Life Technologies, Carlsbad, CA) reagent according to the manufacturer's instructions. SIM-A9 cells were incubated with virus for 24 h and then sorted via FACS on an Aria Fusion, expanded, and subcultured for immune stimulation experiments. Fold OE was verified via RT-qPCR.

Generation of *Phf15* knockout microglia

Phf15 knockout (KO) SIM-A9 cells were generated using the Alt-R CRISPR-Cas9-mediated gene editing system (guide RNA sequence ACTACATCCTGGCGGACCCGTGG) from IDT (Coralville, IA) using CRISPRMAX Lipofectamine reagent (IDT) as per the manufacturer's instructions. ATTO 550+ cells were single-cell sorted on an Aria Fusion. Clones were screened for *Phf15* deletion using PCR (primers Forward: agcacacttgtaaccctcct and Reverse: gaccaatgtctgttgttgttcg) followed by restriction digest with BtgI (New England Biolabs, Ipswich, MA). Percent decrease in *Phf15* mRNA transcript expression was determined via RT-qPCR. Primer sequences are listed in Supplementary Table 1.

Immune stimulation

For all immune stimulation time course experiments, cells (KD, KO, and OE, and respective controls) were subcultured in 24-well plates at a density of 0.05×10^6 cells/well (in triplicate) and stimulated with lipopolysaccharide (LPS; final concentration of 100 ng/mL; Sigma Aldrich, St. Louis, MO), CpG oligodeoxynucleotide (CpG ODN; final concentration of 2.5 μmol/L; InvivoGen, San Diego, CA), or polyinosinic:polycytidylic acid [Poly(I:C); final concentration of 25 μmol/L; Sigma Aldrich, St. Louis, MO] for 1, 6, 12, or 24 h. No stimulation controls received an equivalent volume of sterile 1× PBS (Invitrogen, Carlsbad, CA).

RNA extraction

Mice were sacrificed according to the approved protocol. Brains were quickly isolated and frontal cortical areas were dissected, flash frozen, and stored at -80 °C. RNA was extracted using a bead homogenizer (30 s, setting "5"; Bead Mill, VWR) in Trizol reagent (ThermoFisher, Waltham, MA). Total RNA was extracted using the Direct-zol RNA miniprep kit (Zymo Research, Irvine, CA) according to the manufacturer's instructions. For cell lines, after immune stimulation, media was aspirated and wells were washed 2 × with ice-cold 1 × PBS (Invitrogen, Carlsbad, CA). RNA was extracted using the Direct-zol RNA miniprep kit (Zymo Research, Irvine, CA).

RT-qPCR

cDNA was reversed transcribed from total RNA using the SuperScript™ III First-Strand Synthesis System kit (ThermoFisher, Waltham, MA) following the manufacturer's instructions. RT-qPCR was run using SYBR green (Roche, Pleasanton, CA) on a QuantStudio 6 (ThermoFisher, Waltham, MA) real-time PCR machine. All RT-qPCR primers were specific to the desired template, spanned exon-exon junctions, and captured all transcript variants for the specific gene under study. Ct values were normalized to the housekeeping gene hypoxanthine phosphoribosyltransferase (*Hprt*). Primer sequences used in this study are listed in Supplementary Table 1.

RNA-seq library preparation and analysis

RNA was extracted from a total of $n = 3$ replicates per condition (*Phf15* KO or control) and was used to prepare libraries for RNA sequencing using the mRNA HyperPrep Kit according to the manufacturer's instructions (KAPA Biosystems, Wilmington, MA). Libraries were quality control checked via Qubit (ThermoFisher, Waltham, MA) and via RT-qPCR with a next generation sequencing library quantification kit (Zymo Research, Irvine, CA). RNA sequencing (one lane) was performed on a HiSeq4000 sequencing system (Illumina Inc., San Diego, CA; UC Berkeley Genomics Sequencing Laboratory). Sequencing reads were aligned to the *Mus musculus* reference genome assembly GRCm38 (mm10) using Spliced Transcripts Alignment to a Reference (STAR) aligner[29]. Count data were analyzed with Hypergeometric Optimization of Motif EnRichment (HOMER) software for next-generation sequencing analysis (http://homer.ucsd.edu/homer/ngs/index.html), which uses the R/Bioconductor package DESeq2[30] to perform differential gene expression analysis. To adjust for multiple comparisons, DESeq2 uses the Benjamini-Hochberg procedure to control the false discovery rate and returned false discovery rate adjusted *P* values and $\log_2$-fold expression changes between *Phf15* KO and control conditions for each gene. Genes were filtered by adjusted *P* value (adjusted $P < 0.01$ for upregulated genes or 0.05 for downregulated genes) and $\log_2$-fold change in expression (greater than 1.5 $\log_2$-fold change for upregulated genes and less than -1.5 for downregulated genes). Too few downregulated genes (< 200) passed the more stringent adjusted $P < 0.01$ cutoff for robust downstream biological function analysis, thus the adjusted *P* value threshold was lowered to $P\ adj < 0.05$. Results were visualized using the R package EnhancedVolcano[31]. Lists of upregulated and downregulated genes were input into Metascape[32], a gene annotation and analysis tool, to determine enriched biological themes within the gene lists.

Motif enrichment

Transcription factor binding site ("motif") enrichment was analyzed using HOMER (http://homer.ucsd.edu/homer/ngs/index.html).

Statistical analysis

Relative mRNA expression of *Phf15* in mouse frontal cortical areas was analyzed using ordinary one-way ANOVA with *post hoc* Tukey's multiple comparisons to compare expression levels across age. Percent KD and time course experiments measuring expression levels of inflammatory markers [*Tnfα*, nitric oxide synthase, inducible (*Nos2*), and *IL-1β*] between control and *Phf15* shRNAs sh*Phf15*-1 and sh*Phf15*-2 after

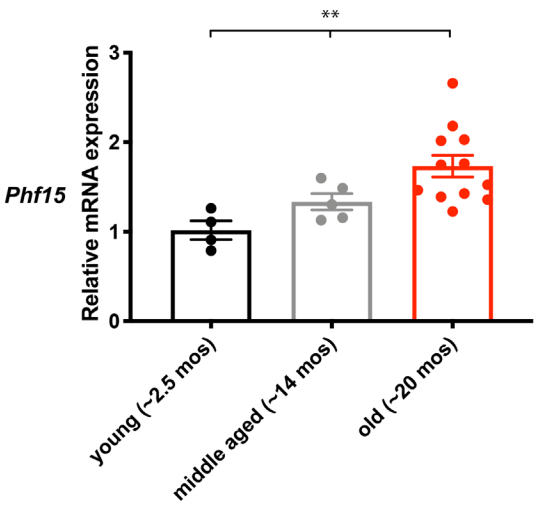

Figure 1. *Phf15* expression increases in aged mouse frontal cortical areas. *Phf15* mRNA expression was significantly elevated in frontal cortical areas of old (~20-month-old; red bar) mice compared to young (~2.5-month-old; black bar) mice. Data are mean ± SEM (n = 4 young, n = 5 middle aged, n = 12 old). One-way ANOVA with Tukey's post hoc comparisons between age groups: $^{**}P < 0.01$

immune stimulation [with LPS, CpG-ODN, or Poly(I:C)] were analyzed via ordinary one-way ANOVA with Dunnett's multiple comparisons between each shRNA versus control shRNA within timepoint. Fold OE or percent reduction for *Phf15* OE and KO cell lines, respectively, were analyzed using unpaired *t*-tests (OE or KO *vs.* respective control). Time course experiments for *Phf15* OE and KO cell lines were analyzed using unpaired *t*-tests with Holm-Sidak correction for multiple comparisons. RNA-seq data were analyzed as described above. $P < 0.05$ was considered significant in all experiments.

RESULTS

Aging increases *Phf15* expression in mouse brain

To investigate whether *Phf15* increases in mouse brains similar to humans[21], we measured *Phf15* mRNA expression in mouse frontal cortical brain areas across age. We were interested in frontal cortical regions because of their involvement in mediating various aspects of cognitive function and because they are selectively affected in several aging-related neurodegenerative conditions, e.g., PD, AD, and frontotemporal dementia[33,34].

We found that compared to young (~2.5-month-old) mice, old (~20-month-old) mice had significantly elevated *Phf15* mRNA levels in frontal cortical areas [Figure 1]. Middle-aged (~14-month-old) mice showed a trend towards increased *Phf15* mRNA expression that did not reach statistical significance. Our data suggest that *Phf15* expression increases in mouse frontal cortical regions upon normal aging, similar to what was previously reported in humans[21].

Knockdown of *Phf15* increases the magnitude of the microglial inflammatory response

To determine whether *Phf15* regulates microglial inflammatory function, we performed loss-of-function studies via shRNA-mediated KD in a murine microglial cell line, SIM-A9, followed by immune activation with LPS, a component of Gram-negative bacterial cell walls and TLR4 agonist. SIM-A9 cells are an established murine microglial cell line and their original characterization has been previously published[35]. In short, SIM-A9 cells are a spontaneously transformed microglial cell line isolated from cultured primary glia from postnatal murine cerebral cortices. They display key characteristics of cultured primary microglia; for example, they express the microglia/macrophage-specific proteins cluster of differentiation 68 (CD68) and ionized calcium-binding adapter molecule 1 (IBA1) and they are responsive to immune

stimulation with LPS, triggering nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) inflammatory signaling cascades, increase of protein levels of the proinflammatory factors NOS2 (the enzyme that catalyzes the production of NO) and cyclooxygenase 2b (COX2), and secretion of TNFα. Importantly, they are also responsive to the anti-inflammatory effects of interleukin 4 and increasing levels of Arginase 1, demonstrating that SIM-A9 cells can switch between pro- and anti-inflammatory states. Finally, they also exhibit phagocytic uptake of bacterial particles and fluorescently-labeled amyloid-β (Aβ).

Additionally, we chose LPS as the immune stimulant because: (1) intraperitoneal and/or intracranial administration of LPS in mice led to increased microglial activation, neuroinflammation, neuronal loss including loss of dopaminergic neurons in the substantia nigra in a mouse model of PD[8], and cognitive and neurological deficits[36]; (2) aged individuals show increased systemic levels of LPS in the bloodstream[37], which are associated with increased inflammation and microglial activation[38]; and (3) in humans, TLR4 activation is linked to age-related pathologies such as PD and AD[39-41]; thus, LPS serves as a relevant aging-related physiological immune stimulant.

KD of *Phf15* resulted in a significant reduction in *Phf15* mRNA transcript levels of 52% and 60% for cell lines sh*Phf15*-1 and sh*Phf15*-2, respectively [Figure 2A], as well as significantly increased mRNA expression of *Tnfα*, a proinflammatory cytokine, after KD with sh*Phf15*-2 at 0, 1, 6, and 12 h after LPS stimulation [Figure 2B].

Similarly, mRNA levels of *Nos2* were significantly elevated at 1, 6, and 12 h post stimulation for sh*Phf15*-2 and 0, 6, and 12 h for sh*Phf15*-1 [Figure 2D]. Overall, our experiments show that ~50%-60% KD, the equivalent of a "heterozygous" condition, results in increased expression of proinflammatory mediators over a 12-h time course that resolves and falls below control levels by 24 h after immune stimulation. Importantly, microglial inflammatory function was elevated in the absence of immune stimulation (0 h time point, Figure 2B and D, and no stimulation condition, Figure 2C and E), suggesting a loss of repressive mechanisms that inhibit basal state inflammatory gene transcription.

We repeated the immune activation time course experiments in *Phf15* KD cells using two separate immune stimulants specific to two distinct Toll-like receptors to test the pathway specificity of the inflammatory response: CpG ODN, a synthetic bacterial and viral DNA mimic that targets TLR9, and Poly(I:C), a synthetic viral dsRNA mimic that targets TLR3. While TLR4 uses both the myeloid differentiation primary response 88 (MyD88) and TIR-domain-containing adapter-inducing interferon-β (TRIF) downstream adapters to transduce its inflammatory cascade, TLR9 and TLR3 utilize MyD88 and TRIF, respectively [Supplementary Figure 1][42,43].

Immune stimulation with CpG ODN and Poly(I:C) both yielded similar results to those obtained with LPS stimulation [Supplementary Figures 2 and 3, respectively], denoting no adapter selectivity and confirming that *Phf15* antagonizes inflammatory gene expression downstream of both the MyD88 and TRIF signaling pathways.

Genetic deletion of *Phf15* increases the magnitude and prolongs the duration of the microglial inflammatory response

Since our KD strategy resulted in ~50% reduction in *Phf15* mRNA expression, we next performed CRISPR/Cas9-mediated genetic deletion of *Phf15* in SIM-A9 microglial cells followed by immune activation with LPS. KO of *Phf15* [Figure 3A] resulted in significantly increased LPS-induced expression of *Tnfα* [Figure 3B], *IL-1β* [Figure 3D], and *Nos2*, albeit to a lesser extent [Figure 3F], over a 24-h time course. Importantly, mRNA levels of both *Tnfα* and *IL-1β* remained elevated at 24 h compared to control cells, denoting a prolonged inflammatory response and failure to return to steady-state. mRNA expression of *Nos2* showed a significant

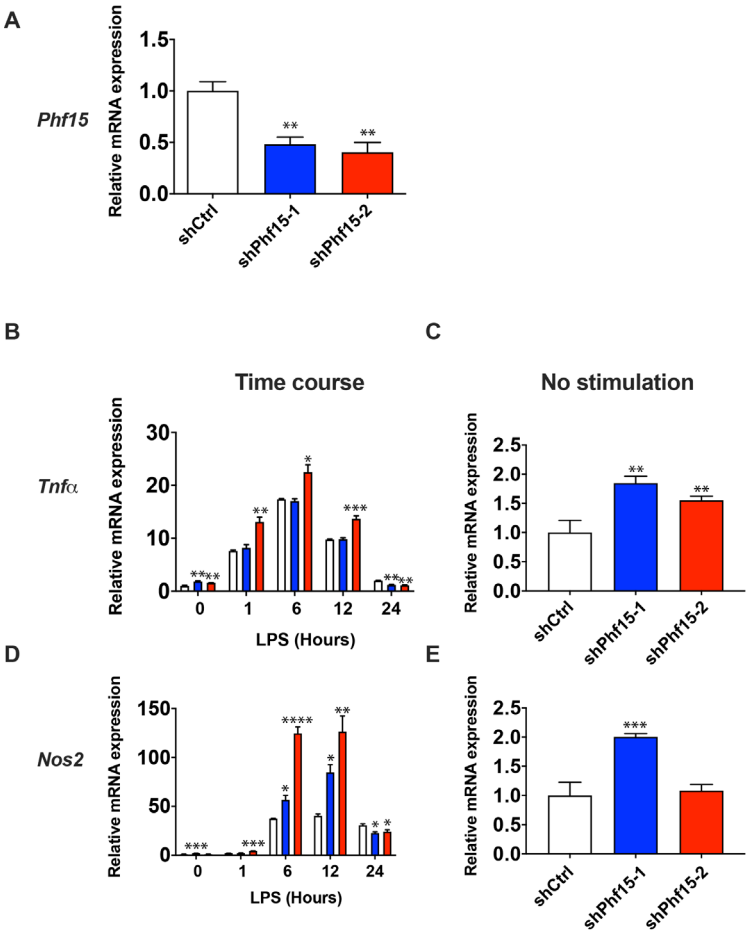

Figure 2. Knockdown of *Phf15* increases the magnitude of the microglial inflammatory response. (A) Knockdown efficiency for anti-*Phf15* shRNAs sh*Phf15*-1 (blue bar, 52% knockdown) and sh*Phf15*-2 (red bar, 60% knockdown). Data are mean ± SEM (n = 3 per condition). One-way ANOVA with Dunnett's multiple comparisons between sh*Phf15*-1 or sh*Phf15*-2 and shCtrl cells: $^{**}P < 0.01$. Twenty-four-hour time course experiments showing relative mRNA expression levels of *Tnfα* (B) and *Nos2* (D) after LPS stimulation of shRNAs sh*Phf15*-1 and sh*Phf15*-2 compared to shCtrl (control scrambled shRNA). No stimulation (0-h time point) is shown for *Tnfα* (C) and *Nos2* (E). Data are mean ± SEM (n = 3 per condition). One-way ANOVA with Dunnett's multiple comparisons for sh*Phf15*-1 or sh*Phf15*-2 relative to shCtrl cells for individual timepoints: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. LPS: lipopolysaccharide; *Tnfα*: tumor necrosis factor alpha; *Nos2*: nitric oxide synthase, inducible

upregulation over 12 h (0-, 1-, and 12-h timepoints) but had returned to control levels by 24 h [Figure 3F]. Notably, basal expression of all three genes was significantly elevated, with a 4-fold increase in *Tnfα*, 14-fold increase in *IL-1β*, and 32-fold increase in *Nos2* when comparing KO to control cells [Figure 3C, E and G].

Time course experiments after stimulation of TLR9 with CpG-ODN [Supplementary Figure 4] and TLR3 with Poly(I:C) [Supplementary Figure 5] in *Phf15* KO cells again yielded similar results to LPS stimulation in *Phf15* KO microglial cells, denoting no difference in downstream adapter selectivity and confirming our prior KD results.

Overall, KO of *Phf15* resulted in a more severe phenotype compared to our KD results, increasing the magnitude and prolonging the duration of the microglial inflammatory response. Taken together, our KD and KO results indicate that *Phf15* functions to restrict microglial inflammatory output, regulating the magnitude and duration, as well as basal inhibition of the inflammatory response.

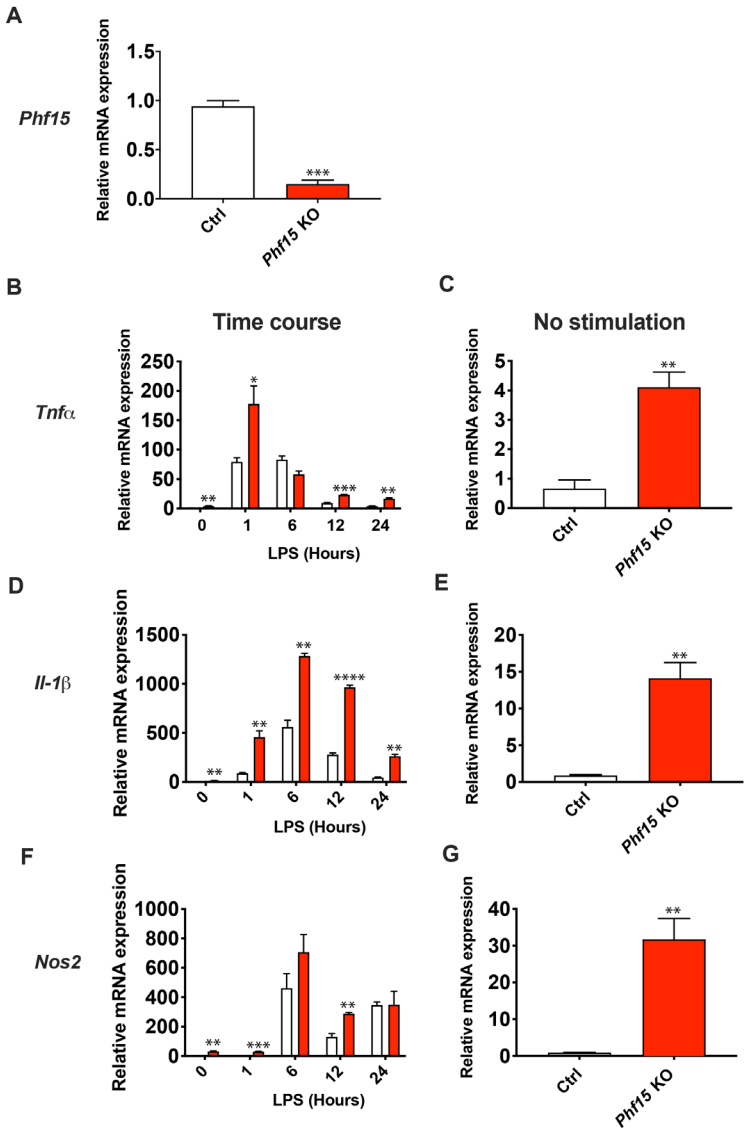

Figure 3. Knockout of *Phf15* increases the magnitude and duration of inflammatory gene expression. (A) Percent reduction in *Phf15* transcript expression in *Phf15* knockout SIM-A9 microglia (*Phf15* KO, red bar) compared to control (Ctrl, open bar). Data are mean ± SEM (n = 3 per condition). Unpaired t-test between *Phf15* KO and control cells. Twenty-four-hour time course experiments showing relative mRNA expressions levels of *Tnfα* (B), *IL-1β* (D), and *Nos2* (F) after LPS stimulation. No stimulation (0-h time point or baseline) expressions of *Tnfα* (C), *IL-1β* (E), and *Nos2* (G) are also shown. Data are mean ± SEM (n = 3 per condition). Unpaired t-tests with Holm-Sidak correction for multiple comparisons between *Phf15* KO and control cells within timepoint: *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. LPS: lipopolysaccharide; KO: knockout; *Tnfα*: tumor necrosis factor alpha; inducible; *IL-1β*: interleukin 1 beta; *Nos2*: nitric oxide synthase, inducible

Overexpression of *Phf15* in microglia results in a dampened inflammatory response

To further test the role of *Phf15* as a repressor of proinflammatory genes, we carried out gain-of-function studies of *Phf15* in SIM-A9 cells. OE via retroviral delivery of the full-length murine *Phf15* cDNA [Figure 4A] resulted in significantly decreased expression of *Nos2* at 0 and 24 h [Figure 4F]. mRNA expression levels of *Tnfα* [Figure 4B], *IL-1β* [Figure 4D], and *Nos2* [Figure 4F] were decreased at 6 h post LPS stimulation at an adjusted P = 0.0501, which did not reach statistical significance. Notably, basal levels (no stimulation) of *Nos2* were also significantly decreased [Figure 4E, G].

Time course experiments following stimulation with CpG-ODN [Supplementary Figure 6] and Poly(I:C) [Supplementary Figure 7] showed a similar but stronger repressive phenotype compared to LPS

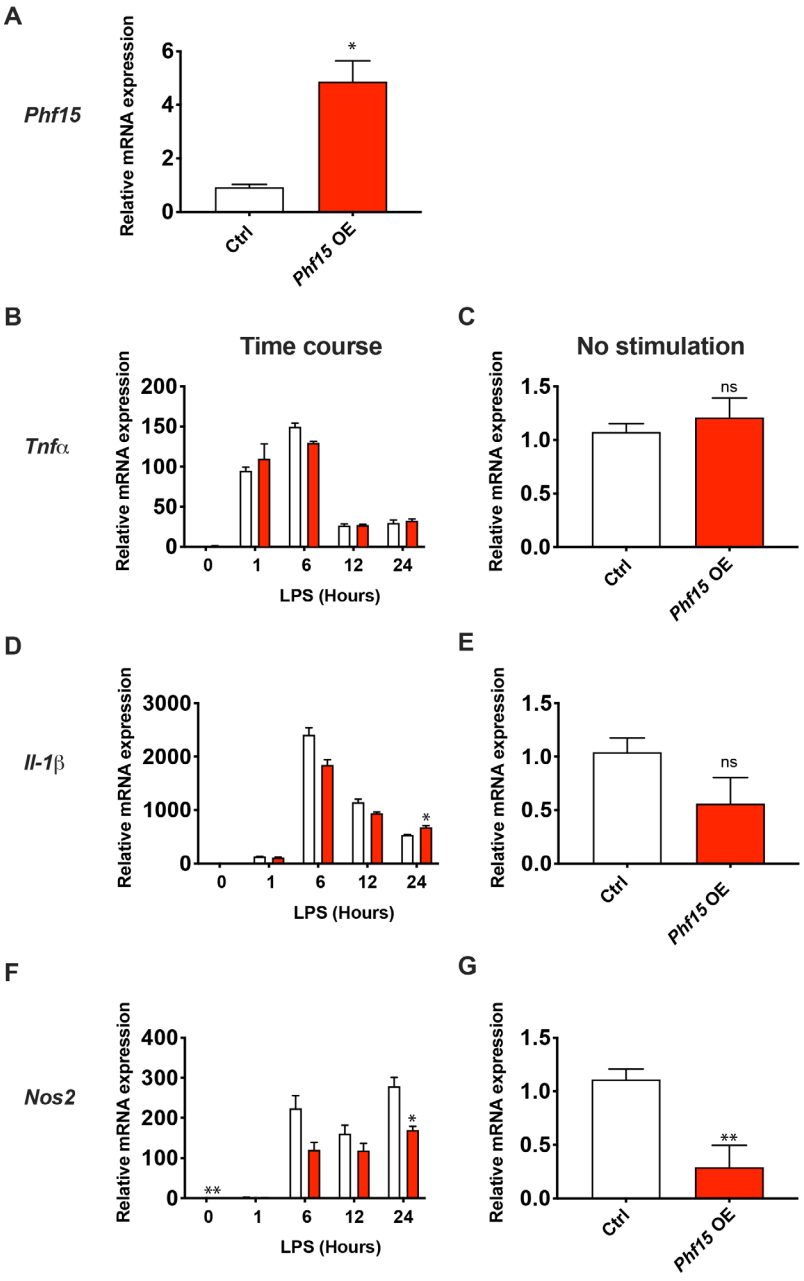

Figure 4. *Phf15* overexpression decreases the microglial inflammatory response. (A) Fold OE of *Phf15* in SIM-A9 microglia (red bar) versus control cells (Ctrl, open bar). Data are mean ± SEM (n = 3 per condition). Unpaired t-test between *Phf15* OE and control cells. Twenty-four-hour time course experiments showing relative mRNA expression levels of *Tnfα* (B), *IL-1β* (D), and Nos2 (F) after LPS stimulation. Baseline (0-h time point, no stimulation) expressions of *Tnfα* (C), *IL-1β* (E), and Nos2 (G) are displayed separately from time course experiments. Data are mean ± SEM (n = 3 per condition). Unpaired t-tests with Holm-Sidak correction for multiple comparisons between *Phf15* OE and control cells within timepoint: *P < 0.05, **P < 0.01. OE: overexpression; LPS: lipopolysaccharide; *Tnfα*: tumor necrosis factor alpha; inducible; *IL-1β*: interleukin 1 beta; Nos2: nitric oxide synthase, inducible

stimulation, with decreased expression levels of *Tnfα* [Supplementary Figures 6A, B and Figures 7A, B], *IL-1β* [Supplementary Figures 6C, D and Figures 7C, D], and *Nos2* [Supplementary Figures 6E, F and Figures 7E, F], over the time course, as well as under basal (no stimulation) conditions, confirming our previous results. Taken together, our OE results show a dampened microglial inflammatory response, revealing a reciprocal response phenotype compared to our KD and KO experiments. Collectively, these results confirm that *Phf15* functions to repress both basal and stimulus-dependent inflammatory gene expressions in microglia.

Loss of *Phf15* affects global expression of genes involved in antiviral responses and regulation of inflammatory processes

To examine global transcriptional changes as a result of *Phf15* deletion in microglia, we carried out RNA-sequencing (RNA-seq) on *Phf15* KO SIM-A9 cells under no stimulation conditions and 6 h post LPS stimulation. We chose to examine the no stimulation condition (0-h time point) based on our KD and KO time course results showing that baseline is one of the most consistently and strongly deregulated time points. Importantly, elevated or "leaky" proinflammatory mediator expression at baseline might result in chronic inflammation leading to neurodegeneration. Similarly, 6 h after LPS stimulation corresponded to the peak of the transcriptional inflammatory response, with large increases in magnitude for both *IL-1β* and *Nos2*.

Differential gene expression analysis revealed that 466 genes with $\log_2$-fold change > 1.5 and *P adj* < 0.01 were upregulated and 309 genes with $\log_2$-fold change < -1.5 and *P adj* < 0.05 were downregulated [Figure 5A]. Biological theme enrichment analysis using Metascape[32] on the upregulated genes revealed that the most enriched biological process categories under basal conditions were "response to virus" and "cytokine production" [Figure 5B and C]. Under the "response to virus" category, there was significant upregulation of various interferon-stimulated genes (ISGs), for example *Isg15*, interferon induced protein with tetratricopeptide repeats 1 (*Ifit1*), *Ifit3*, interferon regulatory factor 7 (*Irf7*), 2'-5'-oligoadenylate synthetase 2 (*Oas2*), and Oas-like 2 (*Oasl2*) [Figure 5C]. The downregulated genes showed more variability in the types of pathways affected, largely involving growth, differentiation, and glial cell migration processes [Figure 5A and Supplementary Figure 8A].

Motif analysis for transcription factor binding sites enriched in the promoters of the upregulated genes at baseline revealed consensus motifs for interferon regulatory factors (IRFs), i.e., the interferon (IFN) stimulated response element (ISRE) and motifs for IRF3 and IRF8 specifically in the top 5 best matches. Activator protein 1 (AP-1) and NF-κB p65 subunit (NF-κB-p65) motifs were also enriched. Both can regulate expression of canonical proinflammatory cytokines such as *TNFα* and *IL-1*β[44,45] [Figure 5D]. Motif enrichment for the set of downregulated genes revealed motifs for twist-related protein 2 (Twist2) and Class A basic helix-loop-helix protein 15 (BHLHA15). Twist2 has been shown to mediate cytokine downregulation after chronic nucleotide-binding oligomerization domain-containing protein 2 (NOD2, a bacterial peptidoglycan sensor) stimulation[46]. BHLHA15 has been shown to induce and maintain secretory architecture in cells specialized for secretion[47] [Supplementary Figure 8B].

Differential gene expression analysis after 6 h of LPS stimulation in KO versus control cells revealed 576 upregulated genes ($\log_2$-fold change > 1.5 and *P adj* < 0.01) and 322 downregulated genes ($\log_2$-fold change < -1.5 and *P adj* < 0.05) [Figure 6A]. Interestingly, by 6 h after LPS administration, some of the most enriched biological process categories in KO cells were related to "cytokine secretion" and "immunoregulatory interaction" [Figure 6B, C], denoting a strong increase in magnitude of expression of genes involved in regulating the secretion of proinflammatory mediators. The downregulated genes at 6 h after LPS stimulation in KO cells relative to control again displayed more variability, but did show decreases in biological process categories related to "regulation of defense response" and "cytokine production", indicating negative regulation of these processes in *Phf15* KO cells compared to control [Supplementary Figure 9A].

Motif enrichment analysis for transcription factor binding sites enriched in the promoters of upregulated genes at the 6-h time point revealed consensus sequences for AP-1, a key regulator of microglia reactivity in inflammation[48] [Figure 6D]. Motif enrichment for the set of downregulated genes revealed motifs for IRFs (the ISRE) and motifs for IRF1 and IRF3 specifically [Supplementary Figure 9B], supporting the observation that there is a negative "regulation of defense response" by 6 h post stimulation. It is interesting to note that a functional transition from cytokine production to cytokine secretion seems to occur in the 6-h period after LPS activation.

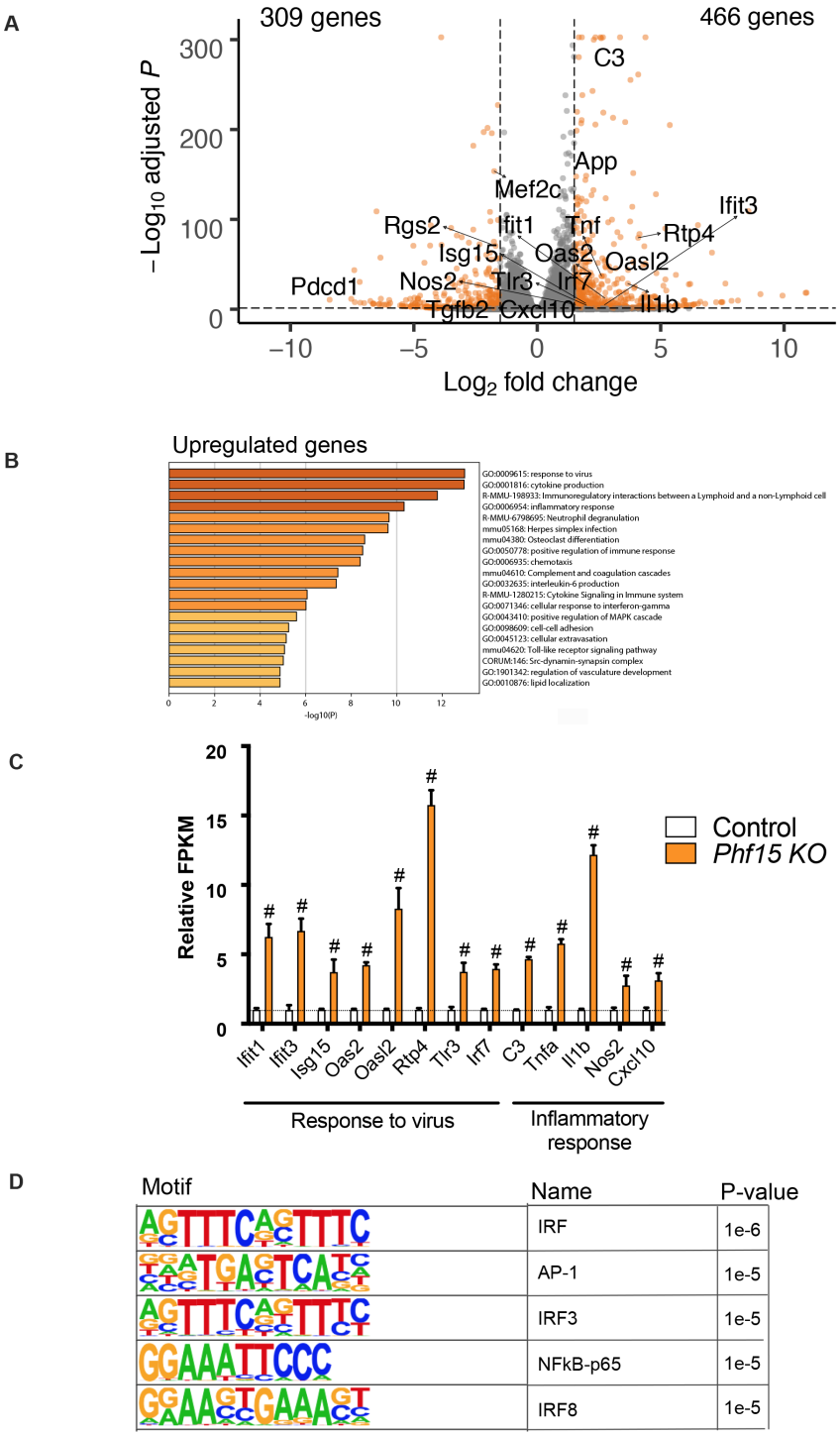

Figure 5. Loss of *Phf15* affects the expression of genes involved in viral response and regulation of inflammatory processes in the absence of immune stimulation. A: Volcano plot representing the RNA-seq results. Orange dots represent differentially expressed genes in *Phf15* knockout microglia compared to control (upregulated genes at a cutoff of $\log_2$-fold change > 1.5 and *P adj* < 0.01; downregulated genes at a cutoff of $\log_2$-fold change < -1.5 and *P adj* < 0.05); B: GO analysis for significantly upregulated genes showing biological process categories related to "response to virus" and "inflammatory response"; C: Upregulated genes associated with "response to virus" and "inflammatory response" in the no stimulation (baseline) condition. Relative FPKM values were obtained by normalizing FPKM values of *Phf15* knockout SIM-A9 microglia to control FPKM values for each gene (n = 3 per condition). Statistics are by DESeq2: $^{\#}P$ < 0.0001; D: Top 5 enriched transcription factor binding motifs for the set of upregulated genes in the no stimulation (baseline) condition. KO: knockout; FPKM: Fragments per kilobase million; GO: Gene ontology

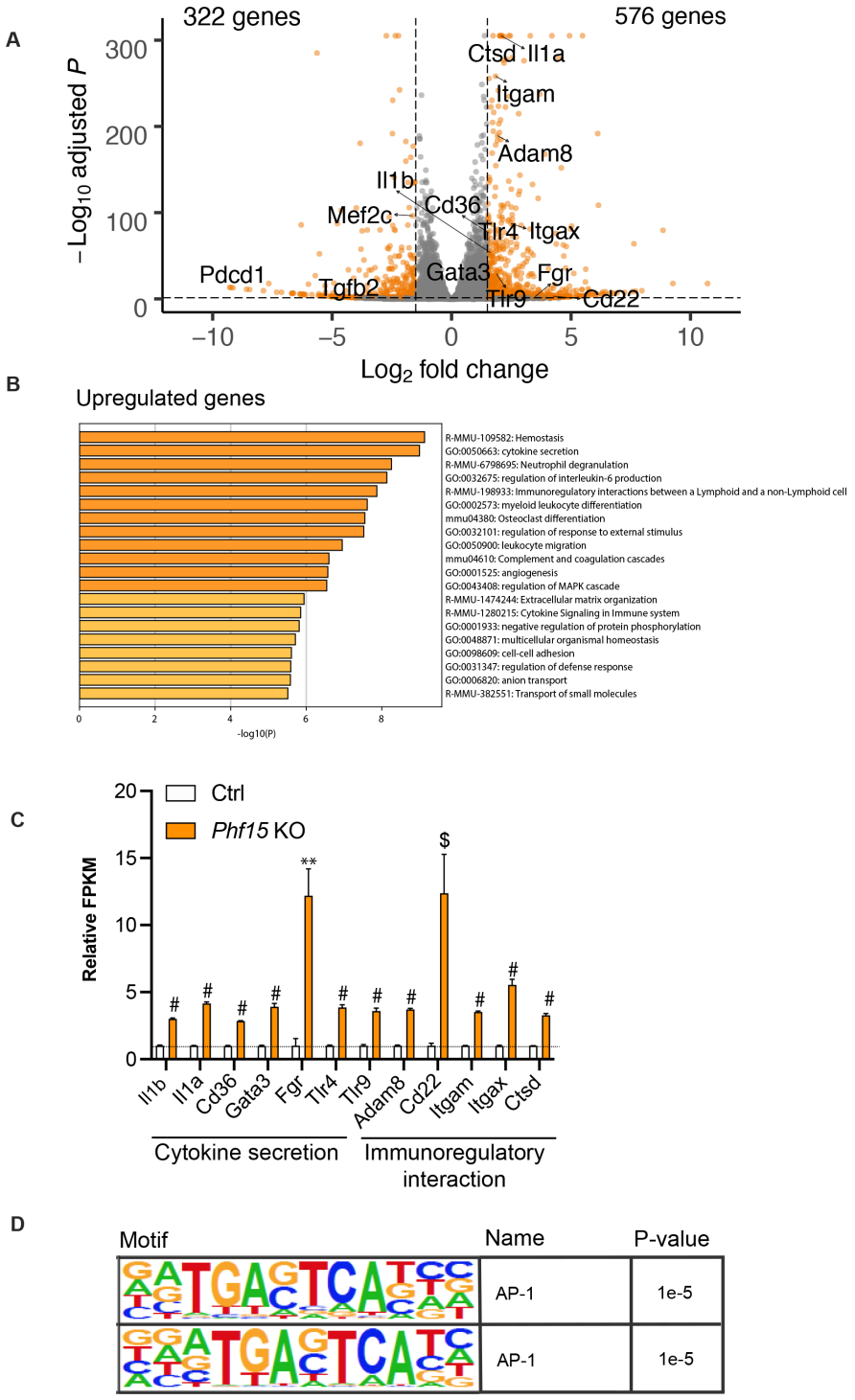

Figure 6. Knockout of *Phf15* affects the expression of genes involved in inflammatory factor secretion and immunoregulatory processes after LPS stimulation. A: volcano plot representing the RNA-seq results. Orange dots represent differentially expressed genes in *Phf15* knockout microglia 6 h after LPS administration compared to control (upregulated genes at a cutoff of $\log_2$-fold change > 1.5 and *P adj* < 0.01; downregulated genes at a cutoff of $\log_2$-fold change < -1.5 and *P adj* < 0.05); B: GO analysis for upregulated genes shows biological process categories associated with "cytokine secretion" and "immunoregulatory interaction"; C: Upregulated genes associated with "cytokine secretion" and "immunoregulatory interaction" biological process categories 6 h post LPS stimulation. Relative FPKM values were obtained by normalizing FPKM values of *Phf15* knockout SIM-A9 microglia to control FPKM values for each gene (n = 3 wells per condition). Statistics are by DESeq2: $^{**}P < 0.01$, $^{\$}P < 0.001$, $^{\#}P < 0.0001$; D: Transcription factor binding motifs for the set of upregulated genes 6 h after LPS stimulation are enriched for activator protein 1 (AP-1). LPS: lipopolysaccharide; KO: knockout; FPKM: Fragments per kilobase million; GO: Gene ontology

Taken together, our RNA-seq results confirm that *Phf15* is a repressor of microglial inflammatory gene expression, regulating the antiviral responses - specifically, IFN-I-dependent responses - as well as processes related to proinflammatory cytokine production and release.

DISCUSSION

Our results show that *Phf15* inhibits microglial expression of proinflammatory mediators under basal and signal-dependent activation, regulating both the magnitude and duration of the inflammatory response. Genetic deletion of *PhfF15* in a microglial cell line followed by stimulation with LPS led to an exaggerated proinflammatory response with increased production of *Tnfα*, *IL-1β*, and *Nos2* over a time course of 24 h. Importantly, levels of proinflammatory factors remained elevated at 24 h, demonstrating a sustained and prolonged response. Consistent with our LPS stimulation of TLR4 results, similar results were obtained after TLR9 and TLR3 activation, confirming that *Phf15* is a general negative regulator and controls both the MyD88 and TRIF downstream signal transduction pathways [Supplementary Figure 1]. Overexpression of *Phf15* showed a dampened microglial inflammatory response, highlighting a reciprocal response phenotype that further supports our loss-of-function results.

Prolonged inflammation can damage surrounding healthy tissue, eventually resulting in neuronal degeneration and loss, and negatively affecting brain function. For example, levels of TNFα are seen to rapidly rise in experimental models of PD and are highly toxic to dopaminergic neurons[13,14,49]. Similarly, high levels of TNFα are a hallmark of PD in humans[50-52]. Additionally, both TNFα and IL-1β are involved in maintaining proper synaptic plasticity at physiological levels[53,54] and overproduction of these cytokines can result in neuronal death via excitotoxicity and cognitive dysfunction[55,56].

Our studies further demonstrate that *Phf15* can regulate both basal and signal-dependent microglial inflammatory gene expression. KD and KO of *Phf15* in microglial cell lines resulted in significantly increased levels of proinflammatory cytokine gene expression - without stimulation and after immune activation - while OE had the reverse effect. The inflammatory response is a tightly controlled process in immune cells in order to protect against unintended damage to healthy tissue. Even in aged microglia, where production and secretion of proinflammatory mediators is generally increased, this process is dependent upon treatment with immune stimulants[9,57,58]. Increased proinflammatory cytokine gene expression without stimulation denotes constitutive or "leaky" expression of inflammatory mediators, simulating a state of low-grade but constant activation. Similarly, hyperresponsiveness to immune stimuli combined with a lack of resolution of the inflammatory response can lead to a state of chronic inflammation. All three can trigger pathological chronic inflammation in the brain, which is detrimental to brain function.

Importantly, distinct molecular mechanisms regulate transcriptional control of different phases ("modules") of the inflammatory response and it is noteworthy that *Phf15* might be involved in regulating several of these. Basal inflammatory function, for example, is generally regulated by co-repressors such as nuclear receptor co-repressor (NCOR), silencing mediator of retinoid and thyroid receptors (SMRT), and RE1 silencing transcription factor (REST) co-repressor 1 (RCOR1 or CoREST) that block poised promoters from active transcription, preventing "leaky" expression of primary response genes (e.g., *TNF*α, Type I IFNs, *IL-1*β, *etc.*) (for review, see[17]). Significantly increased inflammatory gene transcription under baseline conditions, as observed in our *Phf15* KD and KO experiments, suggests a loss of this repressive mechanism.

After stimulation by an activating signal, additional mechanisms can maintain quiescence by restraining active transcription. For example, nuclear receptors such as peroxisome proliferator-activated receptor-γ, glucocorticoid receptor, and liver X receptors can inhibit the signal-activated exchange of co-repressors for co-activators at poised promoters, inhibiting the initiation of transcription[15,17]. Lastly, several mechanisms regulate resolution of inflammation at the transcriptional level, including transrepression

mechanisms that can remove transcription factors such as NF-κB from inflammatory gene promoters, effectively blocking expression of secondary response genes, that is, genes which require chromatin-modification as well as protein synthesis for their induction (e.g., *Nos2* and ISGs)[8,16,17]. Timely resolution of an inflammatory response is crucial in order to limit cellular and tissue damage caused by prolonged or chronic inflammation. Our results suggest that *Phf15* may be involved in regulating all three of the abovementioned mechanisms.

However, how might *Phf15* be involved in regulating transcriptional repression of the inflammatory response in microglia? PHF15 was first described in embryonic stem cells as an E3 ligase that directly targets Lysine-specific demethylase 1 (LSD1, *Kdm1a*) - a key demethylase of histone 3 lysine 4 - for degradation[22]. LSD1 has been identified as a member of the CoREST co-repressor complex[59,60], which is required for transcriptional repression of inflammation in microglia[8]. We therefore initially hypothesized that increased levels of *Phf15* upon aging might lead to decreased levels of LSD1 and increased microglial inflammatory output. Our results, however, demonstrate that *Phf15* itself inhibits microglial inflammatory function; thus, its purported mechanism for inhibition is likely not via degradation of LSD1.

Interestingly, the global transcriptional changes caused by *Phf15* deletion are highly similar to previously reported age-associated transcriptional changes in microglia[9,61,62]. In particular, a study by Deczkowska *et al.*[63], found "immune system process" and specifically "response to virus" among the most highly upregulated biological categories for differentially expressed genes in microglia of young (2-month old) versus aged (22-month old) mice, consistent with our results in *Phf15* KO microglia. Notably, a study by Hammond *et al.*[62], which used single-cell RNAseq to look at microglia profiles throughout the mouse lifespan, found subpopulations in aged (P540) mouse brains which were largely: (1) inflammatory, that is, they upregulated *IL-1β*, *Tnfα*, and other cytokines; or (2) IFN-I-responsive, upregulating *Irf7* and ISGs, particularly *Ifit3*, *Isg15*, *Oasl2*, interferon induced transmembrane protein 3 (*Ifitm3*), and receptor transporter protein 4 (*Rtp4*), compared to younger adult (P100) brains. Similarly, a recent study from the Tabula Muris Consortium[64], which produced a single-cell transcriptomic atlas of 23 tissues and organs across the *Mus musculus* life span, confirmed that microglia in the aged (P540 and P720) brain are enriched for IFN-I-responsive genes and upregulate a similar set of genes including *Ifit3, Irf7, Isg15, Oasl2, Ifitm3*, and *Rtp4*. The genes upregulated by the interferon-responsive microglia clusters in both studies are highly similar to those upregulated in our *Phf15* KO cells under basal conditions [see Figure 5A and C]. Because ISGs can modulate inflammation[23], it is possible that interferon-responsive microglia could play a role in contributing to the inflammatory signature found in the aged brain. Interestingly, among the set of downregulated genes in *Phf15* KO cells at baseline and 6 h after LPS stimulation is myocyte enhancer factor 2C (*Mef2C*). MEF2C is an important checkpoint inhibitor that restrains microglial activation in response to proinflammatory insults and is lost in brain aging via IFN-I mediated downregulation[63,65]. Thus, an increase in *Phf15* expression in microglia during healthy aging could putatively work to counteract not only microglial activation but increased IFN-I in the aged brain as well.

Notably, a recent study by Readhead *et al.*[66] found that several virus species are commonly present in the aged human brain. Among them, human herpesvirus 6A and 7 (HHV-6A and HHV-7) were highly upregulated in the brain of AD patients and were found to modulate host genes associated with AD risk, for example amyloid precursor protein (*APP*) processing. APP is the precursor molecule whose proteolysis forms Aβ and formation of Aβ plaques has long been thought of as the driving force behind Alzheimer's disease[67]. Aβ has more recently been found to have antimicrobial properties[68], conferring increased resistance against infection from both bacteria and viruses[69]. *App* is among the significantly upregulated genes under basal conditions in our *Phf15* KO cells ($\log_2$-fold change = 1.492 and *P adj* < 0.0001; see Figure 5A). Upregulation of *App* due to loss of *Phf15* in mouse microglia is thus consistent with our data showing *Phf15* regulation of the antiviral microglial response.

Altogether, our results show that *Phf15* is a novel repressor of microglial inflammatory gene expression, regulating both the magnitude and time-to-resolution of the inflammatory response. Importantly, *Phf15* also serves to repress baseline inflammatory output in the absence of immune activation. Putatively, increases in *Phf15* during healthy aging could help counteract brain inflammation and protect brain health.

Future studies will determine the mechanism of action of *Phf15*. For example, the identity of its binding partner proteins and its genome-wide binding sites and associated histone marks will be elucidated to determine the specific gene regulatory regions it interacts with (e.g., active enhancers or promoters). Additionally, studies in *Phf15* KO mice will determine whether loss of *Phf15*-mediated repression of proinflammatory factors is sufficient to induce cognitive decline or exacerbate LPS-induced neurotoxicity of dopaminergic neurons in the substantia nigra.

DECLARATIONS

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Authors' contributions

Designed and performed experiments, analyzed data, and wrote the manuscript: Muroy SE
Performed experiments and analyzed data: Timblin GA, Preininger MK
Performed experiments: Cedillo P
Designed experiments and wrote the manuscript: Saijo K

Availability of data and materials

Not applicable.

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

All procedures were approved by the Animal Care and Use Committee of the University of California, Berkeley (Animal Use Protocol AUP-2017-02-9539).

Consent for publication

Not applicable.

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