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Research Article

# Microglial Inflammatory Mechanisms in Stroke: The Jury Is Still Out

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ABSTRACT

Microglia represent the main immune cell population in the CNS with unique homeostatic roles and contribution to broad neurological conditions. Stroke is associated with marked changes in microglial phenotypes and induction of inflammatory responses, which emerge as key modulators of brain injury, neurological outcome and regeneration. However, due to the limited availability of functional studies with selective targeting of microglia and microglia-related inflammatory pathways in stroke, the vast majority of observations remain correlative and controversial. Because extensive review articles discussing the role of inflammatory mechanisms in different forms of acute brain injury are available, here we focus on some specific pathways that appear to be important for stroke pathophysiology with assumed contribution by microglia. While the growing toolkit for microglia manipulation increasingly allows targeting inflammatory pathways in a cell-specific manner, reconsideration of some effects devoted to microglia may also be required. This may particularly concern the interpretation of inflammatory mechanisms that emerge in response to stroke as a form of sterile injury and change markedly in chronic inflammation and common stroke comorbidities.

## How do microglia actually contribute to outcome after acute brain injury?

Because microglia react rapidly to tissue disturbance, the temporal and spatial characteristics of neuropathological events after acute brain injury are well reflected by changes in microglial morphological, transcriptomic- and proteomic phenotypes (Iadecola and Anrather, 2011; Izzy et al., 2019; Beuker et al., 2022; Hochrainer and Yang, 2022; Li et al., 2022; Zheng et al., 2022; Lenart et al., 2023). This has traditionally associated microglial activity and responses with the extent of tissue injury, perfusion deficits, neuronal dysfunction and loss, blood–brain-barrier (BBB) breakdown, and many other readouts assessed in clinical, neuropathological and experimental studies. Due to the availability of comprehensive review articles covering these aspects, in this paper, we will focus on a set of recent studies that may provide insight into the functional role of microglia through cell depletion or manipulation of core microglial pathways and inflammatory mediators. It is important to note that there are several possible reasons behind the highly controversial data available concerning the functional contribution of microglia to stroke outcome. First, only recent technological advances have allowed (to some extent) selective manipulation of microglia as opposed to the large number of papers with correlative observations linking microglial phenotypes with the evolution of brain injury after stroke. While even the latest experimental models are far from optimal, data availability is also insufficient to draw firm conclusions concerning the contribution of specific microglial mechanisms to stroke-related injury. It also remains difficult to find sensitive biomarkers indicative of the functional role of microglia in clinical studies. Second, the nature and evolution of brain injury may be highly brain region- and stroke type-dependent, due to differences in blood supply, collaterals, neurochemical milieu, contribution of brain vs blood-borne inflammatory cells and several other factors, which occur in line with the large regional heterogeneities of microglial phenotypes and responses to injury in different stages after stroke (Denes et al., 2007; Morrison and Filosa, 2013; Grabert et al., 2016; Tan et al., 2020; Beuker et al., 2022; Zheng et al., 2022). Third, while the important housekeeping roles of microglia in maintaining neuronal circuits, the integrity of the neurovascular unit, cerebral perfusion and other processes under physiological conditions are increasingly recognized, the complex interactions between microglia and neurons, astrocytes, pericytes, endothelial cells, oligodendrocytes and other cells markedly change in the injured brain and the mecha-

*Abbreviations:* BBB, blood–brain-barrier; DAMPs, damage associated molecular patterns; IL-1β, interleukin 1β; MCAo, middle cerebral artety occlusion; NLRs, Nod-like receptors; OGD, oxygen-glucose deprivation; PAMPs, pathogen-associated molecular patterns; PRRs, pattern recognition receptors; ROS, reactive oxygen species.

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nisms underlying these interactions are still largely unclear. Last but not least, it is not easy to model in experimental settings how cell autonomus changes in long-lived, self-renewing microglial populations would influence outcome after different forms of stroke, not mentioning the interpretation of such data in the clinical context considering the broad array of comorbidities and systemic inflammatory conditions known to markedly influence microglial phenotypes in patients (Denes et al., 2010b; Hoogland et al., 2015; Wendeln et al., 2018).

## Microglia as damage sensors and inflammatory cells

According to a simplified, previously held view, microglia become rapidly activated after stroke and through the production of proinflammatory mediators, "activated" microglia would exacerbate the brain damage. However, while experimental data support rapid damage sensing and production of inflammatory mediators by microglia after stroke, the available evidence suggests that the net effect of microglia to injury and repair after stroke may be protective rather than detrimental. As such, lessons from microglia depletion studies appear to be in contrast with the observations that blockade of proinflammatory cytokines, their receptors, reactive oxygen species (ROS) and other substances known to be produced by microglia mediate protection in different experimental models of stroke. These controversies are further augmented by the limited availability of microglia-specific interventions targeting these pathways is experimental stroke models, especially those combined with comorbidities, as discussed in detail below.

In fact, microglia rapidly change their morphology, process motility, contacts with neurons and the vasculature after stroke, in line with rapid production of proinflammatory cytokines and chemokines, ROS, proteases and other substances. These occur in parallel with marked changes in transcriptomic and proteomic fingerprints, followed by increased phagocytic- and proliferative activity across the first days after the insult (del Zoppo et al., 2000; Denes et al., 2010b; Iadecola and Anrather, 2011; Swanson et al., 2019; Lenart et al., 2023). These responses are well explained by the remarkable sensitivity of microglia to tissue disturbance, including changes in extracellular ion gradients, plasma proteins that reach the brain parenchyma across the injured blood brain barrier (BBB), ATP, histones, DNA, HMGB1 and other damage associated molecular patterns (DAMPs) released from injured cells (Lenart et al., 2016; Colonna and Butovsky, 2017; Iadecola et al., 2020; D'Alessandro et al., 2022).

One characteristic example for the induction of a proinflammatory response after stroke – as a form of sterile injury – concerns the production of the master regulatory cytokine, interleukin 1β (IL-1β). Since the discovery of Nod-like receptors (NLRs) and the identification of inflammasomes as the major regulators of IL-1β production, several studies have been focusing on their potential roles in CNS inflammatory diseases (Chen et al., 2009; Geddes et al., 2009). NLRs and inflammasomes are intracellular pattern recognition receptors (PRRs) that are predominantly expressed in phagocytic cells like macrophages and microglia, and sense pathogen-associated molecular patterns (PAMPs) and DAMPs, which are highly relevant in the context of stroke (Martinon et al., 2002). Microglia "activation" is a major contributor to CNS inflammation after stroke. Nevertheless, the precise mechanisms of microglia-related NLR functions in stroke are far not understood. In the next chapter below, we briefly elaborate on the main NLR-mediated effects linked with stroke and discuss microglia-mediated actions, whose functional contribution to stroke outcome via NLRs is still largely unclear.

## Role of NLRs in stroke: What is the evidence for microglia-mediated effects?

Evidence indicates that besides the rapid induction of IL-1β mRNA after stroke, elements of the molecular apparatus required for IL-1β production are also induced both *in vivo* and *in vitro*, and blockade of these leads to reduced inflammation. In particular, levels of the inflammasome forming NLRs NLRP3, AIM2 and NLRC4 increase after stroke in the mouse brain. It has also been shown that NLRP3 expression was significantly increased after oxygen-glucose deprivation (OGD) in primary cultured microglia, and silencing with shRNA significantly attenuated caspase-1 activity and cytokine levels (Yang et al., 2014). However, while genetic deletion of AIM2, NLRC4 or the common adaptor protein ASC in mice reduced infarct size, the role for NLRP3 deficiency was not associated with significant protection (Denes et al., 2015). Elevated levels of the mRNA and protein of AIM2 and NLRC4 were also detected after transient middle cerebral artety occlusion (MCAo) in the rat brain. In line with this, infarct size was decreased after the administration of 17β-estradiol (E2) and progesterone (P) after cerebral ischemia; and E2 and P also significantly reduced AIM2 and NLRC4 expression in cortical astrocytes and microglia cells after OGD. This might, at least in part, explain the neuroprotective effect of E2 and P in stroke (Habib et al., 2020).

In the context of microglial function in stroke, it is important to note that inflammasome activation may convey detrimental effects via at least two different ways: while the release of inflammatory mediators in response to DAMPs like HMGB1 could promote brain injury, induction of inflammasome components may also induce pyroptosis of microglia under ischemic conditions. As we will discuss below, lack of microglia on its own is expected to have marked detrimental effects on stroke outcome. Thus, blockade of NLR-related pathways may have highly controversial effects in different models of experimental stroke. For example, in the BV2 murine microglia cell line, OGD resulted in significantly decreased protein expression of NLRP1, NLRP3 and AIM2, while NLRC4, caspase-1 and caspase-8 were increased (Poh et al., 2019). In line with this, the release of "alarmin" molecules (HMGB1, IL-1α) were detected and pyroptosis was increased. siRNA KO of NLRC4 was able to decrease apoptotic and pyroptotic cell death in BV2 cells after OGD. In primary microglia isolated from brain following cerebral ischemia, expression of NLRC4, ASC and IL-1β was also detected. In line with this, MCAo induced pyroptosis and IL-1β/IL-18 release. AIM2 and MEG3 expression (MEG3 can bind miR-485 to maintain AIM2 expression) was upregulated, while miR-485 was downregulated. In MEG3-KO in cells *in vitro*, pyroptosis and inflammation was inhibited, and AIM2, ASC expression as well as caspase-1 activation and the cleaved form of GSDMD was decreased after OGD, while overexpression of MEG3 did the opposite (Liang et al., 2020). Cytosolic double-stranded DNA (dsDNA) is a danger signal that is sensed by nucleic acid-sensing pattern recognition receptors (PRRs). Cerebral ischemia in mice was found to induce the release of dsDNA into the cytosol in Iba-positive microglia, and enhanced the expression of cGAS and AIM2 evetually leading to inflammation. cGAS is a cytosolic DNA sensor that binds DNA directly and induces the production of cGMP. cGMP as a second messenger induces type I IFN production through STING-IRF3 pathway as well as pro-inflammatory cytokine (TNFα, IL-6) secretion via NFκB signaling. The authors found that the cGAS antagonist A151 (a synthetic oligonucleotide) significantly inhibited cGAS and AIM2 expression, as well as gasdermin D (GSDMD), IL-1β and IL-18 secretion, while prevented microglial pyroptosis after MCAo. In line with this, the cGAS inhibitor reduced cytokine secretion, neutrophil infiltration, leading to smaller brain damage and better functional outcome (Li et al., 2020). These examples indicate that most interventions targeting NLRs and related proinflammatory pathways will not only incorporate changes in the levels of proinflammatory mediators, but also alterations in microglial cell death and complex secondary effects, in which the functional role of microglia may be highly context- and model-dependent.

The available data also suggests that inflammasome-related signaling in stroke is highly complex and includes several converging pathways. For example, Histone deacetylases 3 (HDAC3), which modulates

the acetylation state of histone and non-histone proteins, has been raised to play an important role in inflammation after experimental stroke via AIM2. While HDAC3 expression is increased in microglia after MCAo, intraperitoneal injection of a HDAC3 inhibitor RGFP966 (that can cross the BBB) decreased infarct size and AIM2 inflammasome expression. AIM2 KO mice had reduced brain injury compared to wild type mice and the HDAC3 inhibitor failed to protect in AIM2 KO mice. In this study, the HDAC3 inhibitor also attenuated STAT1 phosphorylation and activation, which partially explains the negative effect on the AIM2 inflammasome (Zhang et al., 2020). Erythropoietin (EPO) mitigates TAK1 (TGFβ-activated kinase) expression and activation, also the phosporylation of NFκB. EPO attenutes post-ischemic upregulation of NLRP3/NLRC4/AIM2 mRNA. TAK1 deficiency in microglia and border- associated macrophages (Cx3cr1creER-Tak1fl/fl) was found to reduce infarct size after stroke (Zeyen et al., 2020). In line with this, EPO administration improved clinical outcomes and dampened stroke-induced activation of TAK1 and inflammasomes. TAK1 regulates NLRP3, NLRC4 and AIM2 expression in microglia and brain macrophages following MCAo in Cx3cr1-creER-Tak1fl/fl mice. However, pharmacological inhibition of NLRP3 in microglial BV-2 cells did not influence post-OGD IL-1β levels, but increased NLRC4 and AIM2 protein levels, suggesting compensatory activities among inflammasomes (Heinisch et al., 2022). Tripartite Motif Containing 29 (TRIM29) may function as a neuroprotective molecule. It is upregulated after MCAo and OGD. Deficiency of TRIM29 enhanced apoptosis and pyroptosis in neurons and microglia, as well as NLRC4 activation. TRIM29 was found to directly interact and polyubiquitinate NLRC4 that promotes proteosomal degradation and inhibition of inflammasome activation. Silencing of NLRC4 in TRIM29 deficient mice resulted in decreased IL-1β and IL-18 secretion (Deng et al., 2023).

During sterile inflammation following stroke, inflammasomes may not be the only source of pro-inflammatory cytokines. Non-inflammasome forming NLRs (like NOD1, NOD2, NLRX1, etc.) are also important regulators of the production of cytokines like IL-6 or TNFα. For example, NOD2 expression was enhanced following focal cerebral ischemia in microglia, as also confirmed in primary microglia following OGD. NOD2 activation with the specific NOD2 agonist muramyl dipeptide (MDP) increased infarct size, while deletion of NOD2 lead to smaller infarct size and better functional outcome. NOD2 deletion also reduced TNFα, IL-1β and IL-6 levels in the brain after stroke. Mechanistically, activation of the NADPH oxidase has been revealed as an important part of NOD2-mediated inflammation in this experimental model (Liu et al., 2015). These interesting, but mechanistically complex studies implicate NLRs and inflammasomes in stroke outcome, but the effects concerning the functional role of microglia mostly remain correlative, or indirect. At present, conditional deletion of inflammasome forming proteins and other NLRs from microglia is largely lacking in models of experimental stroke, which studies will be essential to reveal the functional contribution of microglia to stroke-related inflammation and injury, while will also elaborate on the mechanisms of microglial pyroptosis *in vivo*. In this context, selective targeting of microglia and studies on microglial inflammatory pathways are also important, because at present the majority of the NLR literature concerns *ex vivo* studies in monocytes and macrophages. While even different macrophage subsets are known to exhibit fundamentally different inflammatory responses to identical stimuli (Budai et al., 2017; Kovacs et al., 2021), such differences may be augmented by the fact that long-lived microglia show remarkable plasticity *in vivo*, and core microglial phenotypes undergo rapid shifts immediately after isolation of these cells from the brain tissue (Hammond et al., 2019; Marsh et al., 2022). Thus, detailed comparison of microglia and macrophage responses to sterile injury and *in vivo* validation of *in vitro* observations may be vital for a deeper mechanistic understanding of microglial responses in stroke.

## Studies targeting microglia and microglial inflammatory pathways in stroke

Recent studies show that the net effect of microglia on stroke outcome may be protective, while dysfunctional microglia may exert harmful effects in multiple ways in different phases after stroke (Table 1). This diversity of microglial contributions has important

**Table 1**
***In vivo* studies investigating the role of microglia and relevant inflammatory pathways in stroke or stroke comorbidities.** Note that the table lists specific examples to discuss some known limitations of the different approaches used and was not aimed to provide a full spectrum of published papers. Refer to abbreviations and detailed explanation in the text. Studies *not* using experimental models of stroke or ischemia are shown in *Italic*

| Experimental approach | Methods used | Outcomes | Limitations | References |
|---|---|---|---|---|
| Microglia depletion | Pharmacological (CSF1R inhibitors PLX3397; PLX5622) or antibody-based (AFS98 against CSF1R) | Larger infarct size, worse functional outcome, augmented pathological network activity, excessive inflammation, altered astrocyte activity after stroke; *anti-hypertensive effects* | CSF1R inhibitors act on border-associated macrophages in addition to microglia and may also affect peripheral macrophage populations; Long-term microglia depletion may induce compensatory effects in other cells | Hu et al., 2020; Jin et al., 2017; Marino Lee et al., 2021; Otxoa-de-Amezaga et al., 2019; Szalay et al., 2016 |
| Microglia depletion | Cx3Cr1-driven expression of diphteria toxin receptor (R26iDTR) | Larger infarct size, worse functional outcome or reduced infarct size, improved functional outcome | Cx3Cr1 is also expressed by border-associated macrophages and peripheral macrophage populations; Microglia depletion by iDTR may induce higher level of inflammation compared to CSF1R inhibitors | Li et al., 2021; Huang et al., 2023 |
| Blockade of microglial proliferation | Pharmacological (inhibition of CSF1R by ki20227) or genetic (HSV-TK/GCV: transgenic mice expressing a mutant thymidine kinase form of herpes simplex virus driven by myeloid-specific CD11b promoter, induced by gancyclovir) | Larger infarct size, worse functional outcome | Both CSF1R inhibition and CD11b-driven cell depletion may affect other macrophage populations in addition to microglia; Off target effects of the HSV-TK/GCV system should be considered | Hou et al., 2020; Lalancette-Hebert et al., 2007 |

(*continued on next page*)

**Table 1** (*continued*)

| Experimental approach | Methods used | Outcomes | Limitations | References |
|---|---|---|---|---|
| Blockade of microglial P2Y12R | Injection of PSB0739 into the cisterna magna | Larger infarct size, worse functional outcome, augmented pathological network activity, *increased BBB injury, reduced CBF* | While microglia-specific, effects on microglial P2Y12R last only for a couple of hours; Compensatory effects in other cells in the brain may need to be considered. Detailed assessment of CBF and BBB changes in experimental stroke is lacking | Lou et al., 2016; Cserep et al., 2020; Bisht et al., 2021; Mastorakos et al., 2021; Csaszar et al., 2022 |
| Blockade of Cx3Cr1 / CxCl1 interactions | Genetic deletion of Cx3Cr1 or Cx3Cl1 | Reduced infarct size, inflammation and better functional outcome after stroke; *Altered retinal blood flow, increased IL-1β production and microglial phagocytosis in diabetic mice* | Cx3Cr1 is also expressed by border-associated macrophages and peripheral macrophage populations; Constitutive (genetic) deletion of Cx3Cr1 / Cx3Cl1 may induce developmental / compensatory effects in central or peripheral tissues | Cardona et al., 2015, Denes et al., 2008; Fumagalli et al., 2013; Mendiola et al., 2016; Mills et al., 2021; Soriano et al., 2002 |
| Blockade of inflammatory mediators or their receptors without specific deletion in microglia | Genetic- or pharmacological blockade of IL-1, IL-6 or TNFα actions | Mostly improved outcome (IL-1), worse or better outcome (TNFα) or no change in infarct size (IL-6) | Contribution of microglia to the production of targeted inflammatory mediators is difficult to assess. Blockade of proinflammatory pathways may also have different compartment-specific effects in central or peripheral tissues. | Allan et al., 2005; Clark et al., 2000; Denes et al., 2010b; Denes et al., 2011b; Gertz et al., 2012; Iadecola and Anrather, 2011; Lambertsen et al., 2012; Lambertsen et al., 2019; Willis et al., 2020; Wong et al., 2019 |
| Conditional deletion of inflammatory modulators in microglia | Cx3Cr1-driven deletion of TAK1, HDAC3, PGC1α, VPS35, NHE1, M3R, NKCC1, CD36 or CSF1R. Tamoxifen-inducible Cx3Cr1 $^{CRE\ ERT2}$ driver lines were used in most models. | Reduced infarct size, improved functional outcome versus increased infarct size and worse functional outcome depending on the target genes. | Cx3Cr1 is also expressed by border-associated macrophages and peripheral macrophage populations (despite loss of transgene from myeloid cells with short turnover, long-lived macrophages remain affected); Side effects of tamoxifen and compensatory actions in other cells should be considered | Costa et al., 2021; Garcia-Bonilla et al., 2021; Han et al., 2021; Liao et al., 2020; Otxoa-de-Amezaga et al., 2019; Song et al., 2018; Toth et al., 2022; Ye et al., 2019; Zeyen et al., 2020 |
| Blockade of inflammatory pathways in models of systemic inflammation and ischemia | Inhibition of IL-1 or TNF actions by using genetic or pharmacological tools in models of aging, LPS priming, diabetes, obesity, atherosclerosis, or infection combined with experimental stroke | Reduced infarct size, improved functional outcome | Contribution of microglia to the production of targeted inflammatory mediators is difficult to assess; Blockade of proinflammatory pathways may also have different compartment-specific effects in central or peripheral tissues | Denes et al., 2010a; Denes et al., 2014; Liberale et al., 2021; McCann et al., 2016; McColl et al., 2007, 2008; Pradillo et al., 2012; Pradillo et al., 2017 |
| Blockade of inflammatory pathways in models of inflammatory preconditioning and ischemia | LPS preconditioning several days prior to stroke or ischemic spinal cord injury | Protective effect of preconditioning is lost upon microglia depletion, blockade of TNFα, TLR4 or IL-1R1 | No direct microglial effects assessed apart from depletion and microglial IL-1R1 manipulation used in one study | Freria et al., 2020; Marsh et al., 2009; Rosenzweig et al., 2007 |
| Blockade of NLRs / inflammasomes and related pathways | Use of NLRP3 KO, ASC KO, AIM2 KO, NLRC4 KO or NOD2 KO mice, blockade of NLRP3 with MCC950 after stroke; blockade of cGAS with A151; blockade of HDAC3 with RGFP966; blockade of TAK1; *blockade of NLRP3 with MCC950 in hypertension* | Reduced infarct size in AIM2 KO, ASC KO, NLRC4 KO, NOD2 KO; no effect of NLRP3 KO acutely after stroke but protective in recurrent stroke; cGAS inhibition and RGFP966 reduce infarct size and microglial pyroptosis; TAK1 deletion in Cx3Cr1 cells reduces mRNAs of NLRs after stroke; *anti-hypertensive effects of MCC950* | Contribution of microglia to the phenotypes observed is difficult to assess; Studies using conditional deletion of NLRs / inflammasomes in microglia combined with experimental stroke are desperately needed for deeper mechanistic insight. | Denes et al., 2015; He et al., 2020; Heinisch et al., 2022; Hu et al., 2020; Li et al., 2020; Liu et al., 2015; Zhang et al., 2020 |

implications for the development of potential drugs to target microglial dysfunction efficiently and within an appropriate time window. In fact, microglia depletion with the CSF1R inhibitors PLX3397 or PLX5622 exacerbates brain injury after transient or permanent MCAo (Szalay et al., 2016; Jin et al., 2017; Otxoa-de-Amezaga et al., 2019). Inhibition of CSF1R by a tyrosine kinase inhibitor (ki20227) in a mouse model of global cerebral ischemia induced by bilateral common carotid artery ligation also exacerbates neuronal injury and leads to worse functional outcome (Hou et al., 2020), which is in line with the known detrimental effects of blockade of microglial proliferation in other stroke models (Denes et al., 2007; Lalancette-Hebert et al., 2007). Concerning the possible mechanisms involved, the emergence of pathological neuronal network activity, increased calcium load of neurons in penumbral tissues during infarct evolution, increased spine elimination during reperfusion, excessive inflammation, altered astrocyte activity / responses or differences in the recruitment of blood-borne immune cells (e.g. neutrophils, monocytes) into the brain tissue combined with lack of phagocytic removal of these cells have been

suggested to occur in the absence of functional microglia. Among these, the emerging role of microglia in modulating astrocyte inflammatory responses and the fact that elimination of microglia may alter astrocyte function (Elmore et al., 2014; Liddelow et al., 2017; Marino Lee et al., 2021) suggest that some of the mechanisms through which microglia protect against brain injury after stroke may be indirect and also involve actions by other cell types. A possible arguement concerning the interpretation of these studies performed in young and otherwise healthy mice could be that microglial dysfunction and more proinflammatory microglia states that are associated with old age and other neurological conditions could still contibute to making brain injury more severe once stroke occurs. However, microglia depletion by PLX5622 or AFS98, a monoclonal antibody against CSF1R also resulted in inreased infarct size in aged (18–19 month old) mice (Marino Lee et al., 2021).

Another set of studies have targeted the ADP receptor, P2Y12R, which is microglia specific in the brain parenchyma (Haynes et al., 2006). Blocking P2Y12R signaling by intracerebroventricular administration of PSB0739 resulted in increased neuronal calcium load, impaired functional connectivity, larger lesion size and worse functional outcome after tMCAo (Cserep et al., 2020). In addition to microglial actions on neurons, blockade of P2Y12R may also negatively impact on vascular responses via at least two possible mechanisms. First, although microglia depletion by PLX3397 did not appear to change the kinetics of BBB breakdown in a model of experimental stroke, blockade of P2Y12R on microglia leads to augmented BBB breakdown after two-photon focused laser excitation or ultrasound-induced vascular injury (Lou et al., 2016; Szalay et al., 2016; Mastorakos et al., 2021). In line with this, blockade of P2Y12R results in impaired hypercapnia-induced vasodilation, neurovascular coupling and adaptation to hypoperfusion (Bisht et al., 2021; Csaszar et al., 2022), although the impact of these mechanisms to cerebral perfusion changes after stroke remains to be established.

A growing number of studies show that modulation of microglial inflammatory responses markedly alters brain injury and functional outcome after stroke. Most studies with conditional microglial gene deletion to date have used tamoxifen-inducible Cx3cr1$^{creER}$ driver lines. The advantage of using this model is a relatively high level of recombination allowing efficient targeting of microglial pathways. However, Cx3cr1 is also expressed by subsets of border associated macrophages (e.g. meningeal and perivascular macrophages), therefore the involvement of these cells in effects devoted exclusively to microglia cannot be ruled out. Nevertheless, in line with some observations mentioned above, Cx3cr1-driven deletion of transforming growth factor-β-activated kinase 1 (TAK1), Histone deacetylase 3 (HDAC3), PPARγ coactivator (PGC1-α), Vacuolar sorting protein 35 (VPS35), or Na+ / H+ exchanger (NHE1) resulted in smaller lesion and better outcome after experimental stroke, while deletion of muscarinic acetylcholine receptor 3 (M3R), $Na^+$-$K^+$-$2Cl^-$ cotransporter (NKCC1) or CSF1R resulted in increased neuronal death, infarct size and worse functional outcome (Song et al., 2018; Otxoa-de-Amezaga et al., 2019; Ye et al., 2019; Liao et al., 2020; Zeyen et al., 2020; Costa et al., 2021; Han et al., 2021; Toth et al., 2022). Controversies also concern Cx3cr1-driven microglia depletion by diphteria toxin receptor (R26iDTR)-mediated manner as reduced brain injury and improved functional outcome versus increased brain injury and worse neurological outcome were also observed (Li et al., 2021; Huang et al., 2023). It should be noted that diphteria toxin receptor-madiated cell depletion in the brain leads to much higher level of inflammation than microglia depletion by CSF1R antagonists, which eliminate microglia via primarily apoptotic cell death. In line with this, deletion of Cx3Cr1 itself, or fractalkine (its sole known ligand) results in reduced brain injury after stroke (Soriano et al., 2002; Denes et al., 2008; Fumagalli et al., 2013).

Of note, while the absence of functional microglia generally aggravates outcome in different experimental stroke models, blockade of key proinflammatory pathways often conveys protection against brain injury. As reviewed in detail elsewhere, genetic- or pharmacological blockade of interleukin-1 (IL-1) actions, or those mediated by tumor necrosis factor alpha (TNFα) reduces neuronal death and improves functional outcome in most studies, although neuroprotective effects of TNFα via TNF-RI have also been reported (Allan et al., 2005; Denes et al., 2010b, 2011b; Iadecola and Anrather, 2011; Lambertsen et al., 2012; Lambertsen et al., 2019). Although several cell types may produce proinflammatory mediators in the CNS and peripheral organs, microglia are considered main producers of IL-1 isoforms, TNFα, and IL-6 in the brain. However, comprehensive studies with conditional deletion of these key proinflammatory mediators from microglia in the context of experimental stroke are almost completely lacking. Microglial deletion of CD36, which has been implicated in inflammasome activation resulted in reduced ischemic injury, while intracerebroventricular injection of the IL-1β receptor antagonist IL-1RA prior to tMCAo, reduced infarct volume in WT, but not in CD36 KO mice (Garcia-Bonilla et al., 2021). IL-1R1 in neurons and endothelial cells is also known to mediate neuronal injury and perfusion deficits after stroke (Wong et al., 2019). However, whether IL-1 in this case would be derived from microglia and the exact role of microglial IL-1α and IL-1β remains to be established (such studies are currently ongoing in different laboratories using conditional models).

Pleiotropic actions of proinflammatory mediators and possible differences between central and peripheral effects make definite answers to their functional contribution even more difficult to obtain. For example, while microglial repopulation promotes brain repair in an IL-6-dependent manner after traumatic brain injury, infarct size is not different in IL-6 deficient mice after stroke, but IL-6 produced locally by resident brain cells may promote post-stroke angiogenesis and protection (Clark et al., 2000; Gertz et al., 2012; Willis et al., 2020). Lessons from clinical studies do not make the picture clearer either. A recent systematic review showed that higher circulating IL-6 levels in community-dwelling individuals are associated with higher long-term risk of incident ischemic stroke in a linear pattern and independently of conventional vascular risk factors; and plasma IL-6 was also independently associated with poor outcome and post-stroke infection (Bustamante et al., 2014; Papadopoulos et al., 2022). In the CANTOS trial, canakinumab, a monoclonal antibody to neutralize IL-1β (upstream to IL-6) in patients with recent myocardial infarction showed a beneficial effect against a combined cardiovascular endpoint, including stroke (Ridker et al., 2017). Canakinumab also turned out to be protective in a mouse model of experimental stroke (Liberale et al., 2018). While TNFα levels appear to be increased in stroke patients, TNFα gene polimorphisms show controversial associations with stroke incidence with differences seen in asian and caucasian populations (Cui et al., 2012; Niu et al., 2015). However, it is not possible to conclude to what extent circulating cytokine levels prior to or after stroke reflect an underlying systemic inflammatory burden as opposed to any indication to microglial production of these mediators in patients.

## How would microglial contribution to acute brain injury change under chronic inflammatory conditions?

Common stroke risk factors and comorbidities are associated with dysregulated inflammatory responses characterized by increased systemic inflammatory burden. This is associated with augmented neuronal injury, worse functional outcome and impaired recovery after stroke in both patients and experimental animals. On its own, targeting detrimental inflammatory mechansims in old age, atherosclerosis, hypertension, diabetes, obesity and infections emerges as an important therapeutical perspective, which could also contribute to improved clinical outcome in stroke patients present with markedly heterogenous comorbidities that all involve inflammation. In line with this, stroke not only induces acute central and systemic inflammation, but also substantially contributes to the modulation of systemic immune mechanisms beyond the acute phase

that markedly contribute to chronic inflammation, progression of comorbidities and recovery (Mascie-Taylor and Karim, 2003; Denes et al., 2010b; Smith et al., 2013; Simats and Liesz, 2022). Unsurprisingly, the post-stroke innate immune response also markedly differs in young and old animals as much as in patients (Gallizioli et al., 2023). Specifically, aging, the most important single risk factor for stroke is associated with increased circulating levels of IL-1β, IL-6 and TNFα. In line with C-reactive protein (CRP) levels (Alvarez-Rodriguez et al., 2012). Patients with ischemic stroke also show increased TNFα plasma levels, which correlates with worsened short-term neurological outcome and age, while blocking TNFα in aged mice reversed impaired outcome after experimental stroke (Liberale et al., 2021). It has also been demonstrated that blockade of IL-1 actions or its downstream proinflammatory mediators results in protection against (generally augmented) brain injury and impaired functional outcome in experimental models of common stroke comorbidities including atherosclerosis, diabetes or infection (Denes et al., 2010a; Pradillo et al., 2012; Denes et al., 2014; McCann et al., 2016; Pradillo et al., 2017). However, while the IL-1 receptor 1 antagonist (IL-1RA) anakinra has been shown to effectively reduce blood and CSF levels of inflammatory biomarkers such as CRP or IL-6 in patients with good safety profiles, clinical trials in stroke to date have not been powered to consider its effects in patients with high inflammatory burden, not mentioning likely interference with thrombolysis (Galea et al., 2011; Smith et al., 2015; Galea et al., 2018; Smith et al., 2018). In line with this, urokinase plasminogen receptor plasma-guided anakinra treatment of COVID-19 pneumonia (SAVE-MORE) reduced plasma CRP and IL-6 levels with high level of efficacy concerning reduced mortality and improved long-term outcome (Kyriazopoulou et al., 2021).

What would be the impact of systemic inflammation on microglial function in stroke and would it be possible to reverse microglial dysfunction by systemic targeting of increased proinflammatory burden or specific inflammatory pathways? Available experimental data do not provide firm answers to these questions at present. Robust data show that systemic inflammation results in major shifts in microglial activity (Hoogland et al., 2015). Remarkably, peripherally applied inflammatory stimuli induce acute immune training and tolerance in microglia via epigenetic reprogramming that persists for several months (Wendeln et al., 2018). Microglia not only react rapidly to stroke, but microglial responses also change markedly under systemic inflammatory conditions. This concerns alterations in microglial interactions with neurons, blood vessels, production of inflammatory mediators, changes in phygocytic activity, migration and several other features (del Zoppo et al., 2000; Anrather and Iadecola, 2016; Zhao et al., 2017; Haruwaka et al., 2019; Jayaraj et al., 2019; Iadecola et al., 2020; Cserep et al., 2021; Lenart et al., 2023; Xingi et al., 2023). Human positron emission tomography (PET) studies also show that inflammation-related changes occur in line with changes in microglial states in different neurological conditions as also reflected by transcriptomic studies (H et al., 2017; Sousa et al., 2018; Tournier et al., 2020; Nutma et al., 2021). However, at present the availability of data from studies investigating the functional contribution of microglia to stroke outcome in models of systemic-inflammation using microglia-specific targeting of inflammatory pathways is very limited. Data suggest that microglial inflammatory pathways may contribute to common stroke comorbidities in experimental models. For example, blocking the NLRP3 inflammasome with MCC950 or depleting microglia by PLX5622 have similar anti-hypertensive effects in mice (Hu et al., 2020). In a rat model of diabetic retinopathy, blockade of the renin-angiotensin system (RAS) by candesartan reduced impairments in retinal blood flow and capillary constriction, while fractalkine induced constriction of retinal blood vessels via CX3CR1 (Mills et al., 2021). In turn, diabetic mice with CX3CR1 deficiency upregulate IL-1β in the retina and show increased microglial phagocytic and proliferative activity (Cardona et al., 2015; Mendiola et al., 2016).

But what would be the functional contribution of altered microglial states to stroke outcome? Would inflammatory priming make microglia more “proinflammatory” and hence these cells would countribute to increased neuronal loss, impaired BBB function or other pathologies? Or, alternatively, would exposure of microglia to inflammatory stimuli induce tolerance and mediate protection? If so, what effector microglial function(s) would mediate these effects allowing specific therapeutic targeting? Differences in the nature of inflammatory polarisation of microglia, the timing and duration of these challenges, their relationship with the onset of stroke and several other, presently unknown factors should be taken into account to answer these questions. Experimental studies have not yet been able to address the role of specific microglial properties with cell-specific targeting in experimental stroke. However, the effect of inflammatory pathways have been tested and these show markedly different outcomes depending on the timing of inflammatory stimuli compared to the onset of stroke. For example, treatment with bacterial LPS or IL-1β shortly before experimental stroke exacerbates BBB injury and neuronal death via increased neutrophil recruitment and MMP9, while also potentiates cerebral perfusion deficits, and increases microglial IL-1α production alongside with increased brain oedema and marked morphological transformation of microglia (McColl et al., 2007, 2008; Denes et al., 2011a; Szigeti et al., 2015). However, LPS preconditioning (LPS administered 3 days prior to MCAo) results in marked protection against ischemic brain injury via mechanisms that involve TNFα and Toll-like receptor 4 (TLR4) (Rosenzweig et al., 2007; Marsh et al., 2009). In addition, while serial systemic injections of LPS for 4 consecutive days leads to dramatic morphological changes, increased production of IL-1, and enhanced proliferation of spinal cord microglia alongside with increased microglia-endothelial contacts, preoperative LPSx4 provides protection from ischemia-induced neuron loss and hindlimb paralysis in a mouse model of ischemic spinal cord injury in part via IL-1R1-mediated actions on microglia and endothelial cells (Freria et al., 2020). As discussed above, microglia depletion increases brain injury after stroke in both young and old mice, therefore it will be important to test the effect of systemic inflammatory priming and preconditioning in the absence of functional microglia, in addition to conditional deletion of key inflammatory mediators in these experiments. Of note, the protective effect of ischemic preconditioning was found to be eliminated after microglia depletion in a mouse optic nerve white matter injury model (Hamner et al., 2022), suggesting that changes in microglial states may be important to mediate preconditioning effects at least in the case of otherwise unprimed microglia.

Further open questions include the role of compartment specific microglia-neuron and microglia-vascular interactions that are known to be important to modulate neuronal activity, neuronal injury or blood flow in the brain under inflammatory conditions. Systemic inflammation is associated with impaired cerebral perfusion, CSF flow and changes in neuronal activity (Murray et al., 2014; Bisht et al., 2021; Cserep et al., 2021; Manouchehrian et al., 2021; Csaszar et al., 2022; Lenart et al., 2023). However, the contribution of microglia to these processes after stroke with preceding systemic inflammation remains to be established. It would also be important to study how sensing of PAMPs and DAMPs by microglia changes under systemic inflammatory conditions and how this impacts on stroke outcome. For example, while the NLRP3 inflammasome can be activated by diverse simuli such as ATP, endoplasmatic reticulum stress, amyloid β, colesterol crystals, etc., priming stimuli may also be multiple in addition to the commonly used LPS, resulting in upregulated expression of the inflammasome components NLRP3, caspase 1 and pro-IL-1β (Swanson et al., 2019). In a mouse model of diabetic cerebral ischemia/reperfusion (I/R) injury, caspase-1 expression was colocalized with Iba1 expression in the brain. In line with this in BV-2 cells, FENDRR in addition to other pyroptosis-associated proteins and the NLRC4 inflammasome were increased. High glucose followed by I/R in BV-2 cells, overexpression of LncRNA-Fendrr induced the expression of NLRC4 and the secretion of IL-1β and IL-18, while LncRNA-Fendrr promoted NLRC4-mediated pyroptosis in microglia (Wang

et al., 2021). Using a photothrombotic mouse stroke model, it was shown that a single stroke increased AIM2, NLRC4 and NLRP3 mRNA expression, while in recurrent stroke only NLRP3 inflammasome activation was detectable. ASC specks were observed outside of microglia surrounding the infarct area. Mechanistically, after the first stroke, NLRP3 was primed, and ASC was released but it did not effect NLRP3 activation, and NLRP3-KO did not effect the outcome. However, brain damage was more severe after the second stroke compared to the first one, ASC aggregation was larger, and the aggravation by ASC was abolished in NLRP3-KO mice (He et al., 2020). These example may indicate that testing the effects of inflammasome activation on brain injury and inflammation after stroke in otherwise naive mice may underestimate inflammasome-related effects due to the low levels of the key proteins required for inflammasome assembly. These considerations also apply to studies investigating cell–specific mechanisms in experimental models of comorbidities that may influence stroke outcome.

## Conclusions and future prospects

Microglia are unique cellular sensors of injury and tissue disturbance in the CNS and their phenotype changes have long been associated with diverse pathological states. However, the availability of studies, which could specifically link microglia and given microglial effector functions to stroke outcome is limited. Recent efforts show that modulation of microglial states has remarkable consequences to changes in neuronal activity, neuronal injury, vascular function, BBB integrity and inflammation in the brain, which indicates that selective targeting of potentially harmful microglial actions and eliminating the detrimental consequences of microglial dysfunction could be therapeutically effective. However, the impact of microglial actions on brain physiology and pathology appears to be highly context dependent. At present, it is considered a substantial limitation that most studies performed to date could not identify the key targetable disease mechanisms related specifically to microglia, and it remains unclear, how such mechanisms change under different disease conditions. For example, the absence of microglial CX3CR1 results in impaired outcome in models of Parkinson disease and amyotrophic lateral sclerosis, while it conveys protection after experimental stroke (Cardona et al., 2006; Denes et al., 2008). While these controversies appear to be highly frustrating from the point of view of drawing firm conclusions about the role of “good” or “bad” microglial contributions to brain injury, such experimental models also provide unique opportunities to study the impact of given microglial effector functions (i.e. production of inflammatory mediators, phagocytic activity, regulation of neuronal- or vascular responses, etc) and understand their context-dependent roles in different forms of neurological conditions. An additional step from here could be to assess the cell autonomous contribution of microglia to potentially targetable inflammatory- and injury-associated processes together with stroke outcome by using conditional cell manipulation tools combined with pharmacological interventions in models of common stroke comorbidities and systemic inflammation. The wide repertoire of microglial states and functions in development, plasticity, aging, and diseases that were elucidated in recent years suggest that the risk of “categorization spawns expectations” (Paolicelli et al., 2022) may also apply to this research field, and beyond the need for high quality studies, openness to new concepts is also required to develop effective stroke therapies through microglia-specific interventions in the future.

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