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Immune responses in stroke: how the immune system contributes to damage and healing after stroke and how this knowledge could be translated to better cures?

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Summary

Stroke is one of the leading causes of death and disability worldwide. The long-standing dogma that stroke is exclusively a vascular disease has been questioned by extensive clinical findings of immune factors that are associated mostly with inflammation after stroke. These have been confirmed in preclinical studies using experimental animal models. It is now accepted that inflammation and immune mediators are critical in acute and long-term neuronal tissue damage and healing following thrombotic and ischaemic stroke. Despite mounting information delineating the role of the immune system in stroke, the mechanisms of how inflammatory cells and their mediators are involved in stroke-induced neuroinflammation are still not fully understood. Currently, there is no available treatment for targeting the acute immune response that develops in the brain during cerebral ischaemia. No new treatment has been introduced to stroke therapy since the discovery of tissue plasminogen activator therapy in 1996. Here, we review current knowledge of the immunity of stroke and identify critical gaps that hinder current therapies. We will discuss advances in the understanding of the complex innate and adaptive immune responses in stroke; mechanisms of immune cell-mediated and factor-mediated vascular and tissue injury; immunity-induced tissue repair; and the importance of modulating immunity in stroke.

Keywords: cytokines; inflammation; neuroinflammation.

Introduction

Stroke was first described by Hippocrates as a disease of 'struck down by violence' or apoplexy (Greek) over 2400 years ago. Later studies in the mid-1600s identified apoplexy as a vascular disease associated with bleeding in the brain and blockage of main blood vessels. Until the pioneering work of John M. Hallenbeck,[1–4] it was accepted that stroke was a vascular and thrombotic disease. Currently, however, the role of the immune system in stroke-induced injuries and tissue regeneration processes is more appreciated.

Experimental studies in animal models have revealed that innate and adaptive cellular immune responses following ischaemic stroke occur over a time course spanning from minutes to weeks or even months after the injury.[5] Whether these immune responses are beneficial or detrimental to tissue damage and healing is still a controversial question. Supporting the detrimental role for the early inflammatory immune cell responses, it has been

Abbreviations: BBB, blood–brain barrier; CCL, CC-chemokine ligand; CCR, CC-chemokine receptor; CD, cluster of differentiation; CNS, central nervous system; CXCL, CXC-chemokine ligand; CXCR, CXC-chemokine receptor; DAMP, damage-associated molecular pattern; DC, dendritic cell; ICA, internal carotid artery; IFN, interferon; IL, interleukin; MCP-1, monocyte chemoattractant protein 1; MMP, matrix metalloproteinase; PBS, phosphate-buffered saline; pMCAO, permanent middle cerebral artery occlusion; PRX, peroxiredoxin; tMCAO, transient middle cerebral artery occlusion; TNF-α, tumour necrosis factor α; Treg, regulatory T

shown that immune-deficient nude rats have reduced infarct volume after transient middle cerebral artery occlusion (tMCAO) compared with those of the control Sprague Dawley strain.[6] However, different experimental stroke models might have different outcomes. It has been shown that, in contrast to the previous observations, infarct volumes in immune-deficient rats are similar to controls following permanent middle cerebral artery occlusion (pMCAO).

Here, we will address the complex innate and adaptive immune responses involved in influencing ischaemic stroke pathology. We will describe the different types of innate myeloid cells that are involved during ischaemic stroke and the challenges in differentiating brain-resident and peripherally derived cells. Furthermore, we will highlight the diversity of lymphocytic phenotype, the myriad of factors that they can produce, and how antigen specificity can influence their impact on ischaemic stroke pathology. Overall, we aim to shed light on the molecular mechanisms of immune-induced tissue injury and functional repair following ischaemic stroke with the hope that this information might lead to beneficial immunomodulatory treatments in stroke.

Innate immune responses to ischaemic injuries within the central nervous tissue: microglia provide the first level of defence within the ischaemic brain

Microglia are a type of resident myeloid cell in the central nervous system (CNS) derived from yolk-sac progenitors.[7] After neurodevelopment, microglial cells serve as the regulators of homeostasis in the CNS and are thought to play a variety of roles in neuronal injury and survival.[8] Besides the presence of some brain-resident macrophages, microglia are the majority of myeloid cells in the CNS during steady-state conditions.[8,9] In response to disease or injury, other myeloid cells such as bone-marrow-derived monocytes are recruited to the CNS from the periphery and may exhibit similar morphology and expression patterns to microglia.[10,11] This has made it difficult to distinguish resident microglia from other myeloid cells and to delineate the specific roles of microglia in CNS disease and injury. Current methods of microglial identification rely on cell surface marker expression, morphological distinctions or bone marrow transplantation. The combination of cell surface markers and novel technologies such as two-photon imaging, cytometry by time-of-flight (CyTOF; Fluidigm, San Francisco, CA), cytometry, and whole-genome transcriptomic and epigenomic analysis using bioinformatics has led to a better understanding of microglia functions during stroke. Analysis of cell surface marker expression of CD45 intermediate cells ($CD45^{int}$) by flow cytometry or morphological distinctions with immunohistochemistry are frequently used to identify microglia populations.[12] CD45, also known as protein tyrosine phosphatase receptor type C, is an enzyme that is a member of the protein tyrosine phosphatases family. Due to the existence of various isoforms of CD45, the application of correct anti-CD45 antibody is crucial in the characterization of these cells. Another challenge is that CD45 expression and cell morphology can change during pathological states, indicating that these methods may not be reliable markers for microglia detection. The use of bone marrow chimeric mouse models is problematic as well. For this method, mice undergo irradiation to eliminate resident bone-marrow-derived cells and are then injected with fluorescence-labelled bone marrow from a donor mouse. This allows for distinction between host-derived microglia and donor bone-marrow-derived myeloid cells.[13] However, irradiation therapy prompts inflammatory responses[14,15] and may obscure the ability to define specific microglial roles in stroke. Recently, the discovery of new microglia-specific markers, such as transmembrane protein 119 (Tmem119), has helped to distinguish microglia contributions from those of other myeloid cell populations.[10]

The primary inflammatory responses by microglia are to clear up debris and repair injured tissue. However, they may also promote secondary inflammation-associated damage as multiple studies have indicated that microglia serve as pro-inflammatory responders and increase damage following stroke.[16–19] Specifically, evidence suggests that microglia contribute to increased neurovascular breakdown and permeability by prompting activation of platelet-derived factors,[17] and up-regulating expression of matrix metalloproteinases (MMPs) such as MMP-9,[18] during the early stages of stroke. Additionally, reactive microglia are substantial producers of pro-inflammatory mediators such as tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β), reactive oxygen species and inducible nitric oxide synthase following stroke.[16,19] The CR3 complement receptor, CD11b/CD18 is also frequently used as a microglia marker. The morphology of $CD11b^{+}$ $CD45^{int}$ microglia following stroke is highly dynamic and can produce varying levels of TNF-α and IL-1β in a time-dependent fashion (Fig. 1). These inflammatory factors are thought to contribute to post-ischaemic neuronal damage and apoptosis, so worsening the outcome after stroke.[16] Furthermore, genetic or pharmacological inhibition of certain microglial functions, such as proliferation and release of cytokines, may result in dampened inflammatory responses and neuronal damage after ischaemic insult.[20–23] Importantly, factors such as Fas ligand released by ischaemic neurons may induce a specific pro-inflammatory phenotype in microglia.[24] A recent study by Meng *et al.* suggests that neuronal soluble Fas ligand promotes microglial polarization to the 'M1' classical activation phenotype after cerebral ischaemia;

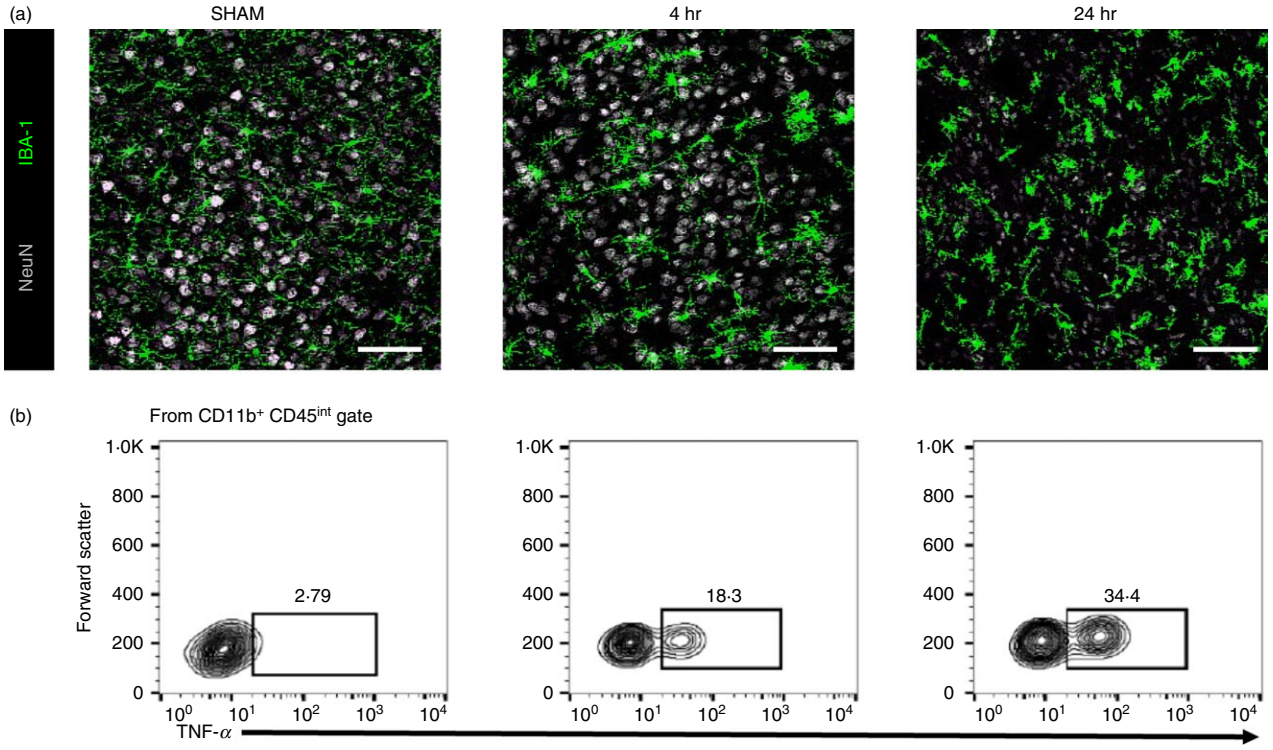

Figure 1. Myeloid cells damage neurons following brain ischaemia. (a) Representative immunohistological staining of myeloid cells (IBA-1) and neurons (NeuN) in the ipsilateral transient middle cerebral artery occlusion (tMCAO) brain in SHAM mice (left), at 4 hr tMCAO (middle) and 24 hr tMCAO (right). Scale bar = 20 μm. (b) Representative flow cytometry staining of microglia ($CD11b^+$ $CD45^{int}$) and their relative expression of tumour necrosis factor-α (TNF-α) in the ipsilateral tMCAO brain in SHAM mice (left), at 4 hr tMCAO (middle) and 24 hr tMCAO (right).

and that 'M1-microglia' release pro-inflammatory factors leading to reduced cell viability and survival.[24]

Findings from several studies already suggest a potential anti-inflammatory role of microglial cells that may contribute to neuroprotection after stroke. Microglial expression of P2X7 receptor,[25] triggering receptors expressed on myeloid cells (TREM2),[26] ST2/IL-33,[27] and B-cell activating factor[28] could be involved in limiting ischaemic neuronal injury. Activation of these microglial-dependent signalling factors results in the release of anti-inflammatory cytokines, such as IL-10, IL-4 and transforming growth factor-β, which may contribute to neuronal survival.[26,27] Microglial secretion of these neuroprotective factors, as well as phagocytic activity, are characteristic of the protective 'M2' phenotype of myeloid cells.[29] Notably, polarization of microglia to an 'M2' state may be driven by signals such as IL-10 and IL-4 from damaged neurons and regulatory B cells after stroke.[28,29] Accordingly, current research focuses on the discovery of methods to induce a switch from pro-inflammatory to protective phenotype in myeloid cells.[29–31] In fact, a study by Narantuya *et al.* suggests that microglial transplant enhances neuroprotection through production and release of neurotrophic factors and M2-related anti-inflammatory cytokines from these donor microglia.[31] Cautionary concerns were also raised regarding microglia polarization and characterization into M1 or M2 phenotypes.[32] It appears that microglia phenotype after stroke is dynamic and influenced by the local environment. Although it was found that MI/M2 polarization is concurrent following traumatic brain injury,[33] our understanding regarding dynamic microglia activation is still limited. A deeper understanding of complex microglia responses to stroke would be critical for developing novel therapies.

Although resident microglia are an attractive target for stroke treatment, null findings from studies suggest that microglia may not be directly involved in worsening the outcome after stroke.[34,35] For example, a study by Harrison *et al.* reports that blocking the major signalling pathway of fractalkine-CX3CR1 between neurons and microglia[36] does not directly contribute to ischaemic damage or protection. Conflicting results from other studies on microglia-specific deficiency of CX3CR1 further complicate our current understanding of microglial roles in cerebral ischaemia.[30,37] Importantly, Tang *et al.* indicate that infiltrating monocytes/macrophages also express CX3CR1 and may be a major source of neuroinflammation and secondary damage following ischaemia. This could account for the contrasting findings, and further emphasizes the importance of distinguishing myeloid

cell populations. In summary, the knowledge of specific roles of microglia following cerebral ischaemia is currently incomplete and requires more study. The ability to distinguish between resident microglia from infiltrating monocytes will be crucial in research that aims to target myeloid cells as a therapeutic strategy against stroke.

Myeloid cells from the periphery respond to stress signals released from the ischaemic brain

Early responses against danger-associated molecular patterns following ischaemic injuries in the CNS are mediated with the remarkably diverse groups of innate immune cells developed within the myeloid cell pathway. The differentiation of these cells is controlled by transcription factors, transcriptional co-regulators and post-transcriptional mechanisms, which have been recently reviewed by others.[38] These 'early' cells rush to the danger site to restore homeostasis and assist in repair. Along with these desirable functions, there are also damage-associated molecular patterns (DAMPs) -induced cellular activities that are detrimental to the tissue. Nonetheless, our understanding about the contribution of innate myeloid cells to beneficial and detrimental responses within the CNS following ischaemic stroke is still limited.

How innate myeloid cells are activated by DAMPs through pattern recognition receptors is a subject of intense study.[39,40] These receptors not only recognize pathogen-derived molecules, but also endogenous molecules that are released in stressed tissues. It is well known that the release of DAMPs from dead cells in ischaemic tissues induces sterile inflammation that contributes to pathogenesis.[41] There are several well-characterized DAMPS in the CNS such as high mobility group box 1 (HMGB1), the S100A8 and S100A9 (S100A8/A9) proteins, and the peroxiredoxin (PRX) family proteins. One of the earliest DAMPs after stroke is the HMGB1,[42,43] which contributes to the damage of the blood–brain barrier (BBB).[44,45] The S199A8/A9 proteins can be produced by infiltrating cells in the brain[46] and contribute to inflammation post-injury.[47,48] Finally, the role of PRX family members is more controversial, but they are known to contribute to numerous tissue injuries.[49–53] Dying cells release PRX, inducing immune cell activation through Toll-like receptor 2 and Toll-like receptor 4 receptors. This leads to the production of pro-inflammatory mediators including IL-1β and IL-23. Interestingly, PRXs can also contribute to neuroprotection following ischaemia.[54] More recently, it was shown that clearance of DAMPs by mononuclear phagocytes through *Mrs1*, *Marco* and *Mafb* gene-regulated mechanisms contributes to tissue protection following stroke.[55] This supports that acute tissue damage following stroke triggered by DAMPs exacerbates tissue injury and contributes to the induction of T-cell-mediated inflammation.[53,56,57] The impact of ischaemic neurons on local and peripheral immune cells is summarized in Fig. 2.

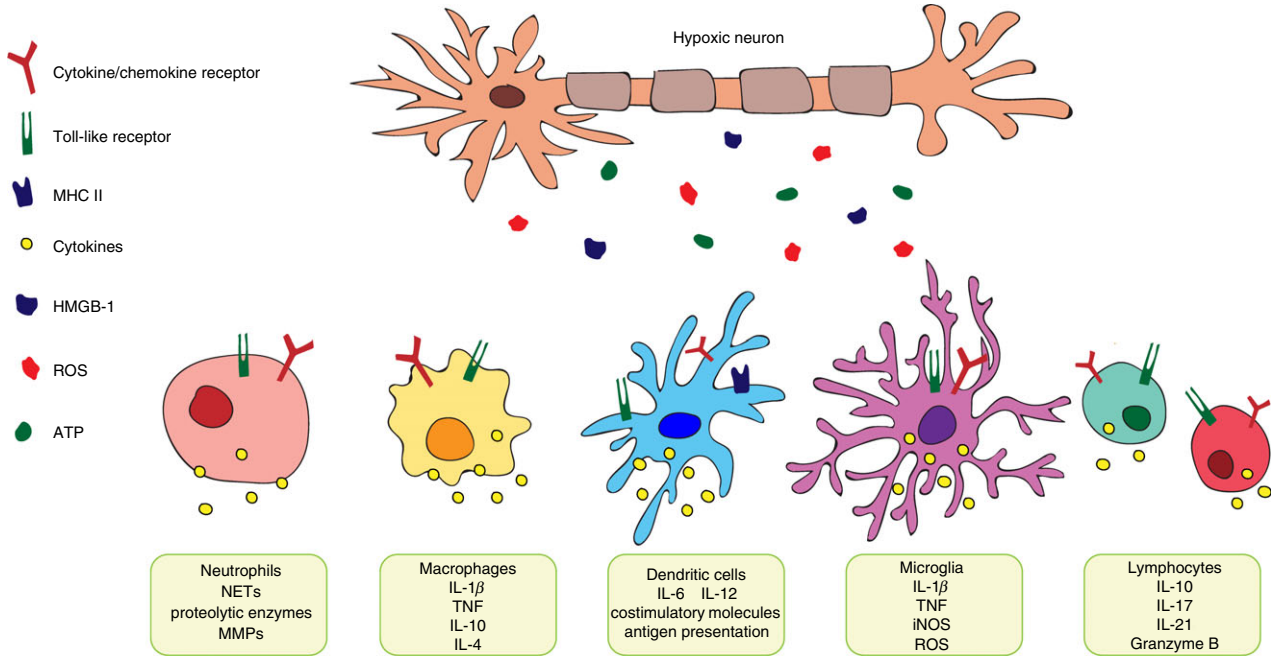

Figure 2. Influence of hypoxic neurons on immune cells. Following brain hypoxia, ischaemic neurons release damage associated molecular patterns such as reactive oxygen species (ROS), ATP, and high mobility group box 1 (HMGB1). This effect can influence local microglia and infiltrating leucocytes to produce inflammatory cytokines to further influence stroke pathology.

There are multiple subtypes of innate myeloid cells including circulating monocytes, tissue macrophages, dendritic cells (DCs), circulating granulocytes (neutrophils, basophils and eosinophils) and mast cells. Circulating granulocytes, specifically neutrophils, are considered as the first infiltrating immune cell type within the stroke-affected tissues. However, the contribution of these cells to tissue damage or regeneration after ischaemic injuries of the CNS is still controversial. The essential immune function of neutrophils in protection against pathogens and their short lifespan demand their constant production and release from the bone marrow. In humans, there are about 10^{11} neutrophils produced daily in a process known as granulopoiesis. This process is highly regulated during conditions of tissue injury and stress.[58,59] Granulocyte colony-stimulating factor has been shown to be the major regulator of neutrophil trafficking from the bone marrow to the blood.[60] In addition, controlled neutrophil mobilization has been shown from the bone marrow to the blood through induction of CXCR2 ligands on these cells.[61] Anti-neutrophil antibodies block the efflux of neutrophils from the bone marrow and inhibit their infiltration into the stroke tissues, which has been shown to be protective in a rat model of hypoxic injury.[62]

Due to early neutrophil infiltration into ischaemic brain tissues, neutrophils were considered as cargo vehicles to deliver macromolecular drugs to block reactive oxygen species-mediated apoptosis following stroke.[63] Clinical studies demonstrated that the neutrophil-to-lymphocyte ratio might be a strong prognostic marker in acute ischaemic stroke[64] and that neutrophil extracellular traps are increased in patients with stroke.[65] Neutrophil extracellular traps are produced by activated myeloid cells, including neutrophils, as networks of DNA, histones and proteolytic enzymes, and are capable of activating platelets and contribute to the thrombotic processes.[66] Taken together, these data indicate that early neutrophil infiltration is detrimental to ischaemic tissue injury following stroke and call for novel therapies to target neutrophil infiltration.

Peripheral monocytes can be recruited to the ischaemic brain within hours after insult as the BBB becomes compromised. As mentioned previously, there is some difficulty in identifying and differentiating monocytes from local microglia based on their similar morphology and phenotypes. However, transplantation techniques and assessment of relative expression of various surface markers have allowed researchers to distinguish peripherally derived myeloid cells from microglia with more confidence. These studies have identified that the two cell types are functionally very different. Further discussion of this subject will continue in the next section. One model to differentiate the two cell types uses the CX3CR1GFP/$^{+}$ CCR2RFP/$^{+}$ bone marrow chimeric mice. These studies have indicated that peripheral CCR2^{+} Ly6C^{high} monocytes infiltrate the ischaemic brain to potentiate stroke damage.[67] Interestingly, there is evidence that these cells could differentiate into microglia-like CX3CR1^{-} Ly6C^{lo} cells that participate in the post-stroke repair process.[13,68]

One of the major cytokines involved in monocyte recruitment to the ischaemic brain is monocyte chemoattractant protein 1 (MCP-1), also known as CCL2. Several groups have characterized the expression of MCP-1 in the stroke brain identifying it in the cortex and mainly expressed on brain endothelial cells or astrocytes.[69–71] Evidence suggests that MCP-1-deficient mice are protected from stroke following pMCAO due to the limited amount of recruited monocytes in the stroke brain as well as the lower levels of IL-1β production compared with littermate mice.[72] Interestingly, in CCR2-deficient (the cognate receptor for MCP-1) mice that have reduced numbers of infiltrating macrophages in the stroke brain, Schilling *et al.* was not able to detect a difference in infarct volume compared with littermate controls. This suggests that the receptor may have conflicting effects depending on whether it is expressed on microglia or macrophages.[73] Aside from MCP-1, macrophage inflammatory protein 3α, also known as CCL20, has been suggested to be involved in pro-inflammatory macrophage recruitment to the ischaemic brain and subsequent TNF-α and IL-1β production.[74–76]

There is also evidence that macrophages can be anti-inflammatory following stroke. Chu *et al.* observed that Ly6C^{high} monocytes can exert a protective effect following tMCAO by polarizing to an M2 phenotype based on local damage signals.[77] Furthermore, Liu *et al.* showed that IL-4 is important for M2 phenotype induction and suggested IL-4 therapy as a potential approach for long-term functional recovery after stroke.[78] In addition, Korhonen *et al.* indicated that IL-33 administration could steer local macrophages to produce T helper type 2 cytokines such as IL-4, and induce stroke recovery.[79] Lastly, it was also shown that macrophages could produce growth factors such as basic fibroblast growth factor, glial cell-derived neurotrophic factor, insulin growth factor 1, and vascular endothelial growth factor to promote functional recovery following stroke.[80]

Dendritic cells are the bridge to adaptive immunity following ischaemic stroke

Dendritic cells are an adaptive immune cell recognized for their antigen-presentation capability and importance in facilitating a T-cell response during disease. Multiple groups have shown that following acute cerebral injury in both rats and mice, there is an increase in the number of infiltrating DCs into the CNS.[81,82] In the murine stroke models, DCs are visualized in both the core infarct region

as well as the border region. Interestingly, bone marrow transplantation experiments have helped to identify how DCs originate, giving us a clue as to how they function. DCs from the periphery mainly reside in the core infarct area whereas resident DCs lie in the border region.[81] In addition, these dendritic cells have an increased expression in major histocompatibility complex II and co-stimulatory molecule CD80, suggesting that there is important T-cell interaction occurring in these areas.[81] Moreover, pMCAO in rats presents similar results. An increase in DC number is observed as early as 1 hr following pMCAO, remains elevated for 24 hr, and increases over the next 6 days. To assess the functionality of these DCs, *in situ* hybridization has been performed to detect cytokine mRNA expression in the infiltrating DCs. At 1 hr following pMCAO, the mRNA levels of IL-1β, IL-12, IL-6, IL-10, TNF-α and interferon-γ (IFN-γ) in DCs were increased compared with DCs in both sham controls and in the non-ischaemic hemisphere.[82] The positive correlation of the number of DCs and their cytokine-producing potential between the infarct volume suggests a damaging role for DCs following stroke in rodents.

In humans, however, the role of DCs in ischaemic brain injuries is not so well-described. One group found that following stroke in human patients, the relative and absolute number of DC precursors in circulation is decreased compared with healthy controls. They show that this effect only lasts for a few days, with the number of DC precursors soon returning to normal. Interestingly, patients with the lowest levels of DC precursors in circulation had larger infarct sizes assessed through computed tomography scan. To further investigate this, post-mortem brain tissue was analysed for the presence of DCs and T cells. Patients with larger infarct sizes had more DC–T-cell clusters compared with patients with smaller infarcts, and they were located near intracerebral vessels. These data suggest that following cerebral injury, DC precursors quickly leave the circulation and infiltrate the CNS to interact with other infiltrating immune cells.[83]

Many groups have shown that DCs infiltrate the CNS following stroke in both rodents and humans. Recent work has exploited these findings to develop new therapies for stroke. By deriving DCs *ex vivo* and engineering them to express specific proteins of interest, it allows for a novel way to administer pharmaceuticals following stroke.[84] Works *et al.* expanded on these findings to derive DCs to over-express soluble TNF receptor 1, which ultimately blocks TNF-α bioavailability. They injected these DCs into rats who had undergone tMCAO 6 hr earlier and saw reduced infarct volume compared with rats who received DCs expressing GFP lentivirus.[85] Using the quick infiltration of DCs and the prolonged time they reside in the CNS may provide a unique and novel approach to drug delivery in human stroke patients.

Lymphocytes encompass adaptive immune-mediated responses following ischaemic stroke

Recently, T lymphocytes have been identified to play a major role in acute ischaemic stroke pathology. Within hours after tMCAO there is an influx of T lymphocytes, which aggregate around the border of the infarcted region. Particularly, cytotoxic $CD8^+$ lymphocytes have been indicated to be recruited into the stroke brain as early as 3 hr following stroke while $CD4^+$ T cells and natural killer cells are recruited within 24 hr and peak at 72 hr post-reperfusion.[5,86] In order to further characterize the timeline of T-cell infiltration to the stroke brain, novel techniques such as multiphoton laser scanning microscopy have been applied to monitor immune responses in real time *ex vivo*.[87–89] The first study by Stoll's group described the spatial and temporal infiltration of T cells following MCAO.[90] Since these studies, there is strong support for the role of immune cells in promoting inflammation that cause secondary tissue injury in the brain following stroke.[91] T-cell infiltration is becoming increasingly acknowledged as a key mediator of the acute phase of stroke pathology in both human disease and rodent models.[92] It was shown that in murine models of both permanent and temporary cerebral ischaemia, T-cell populations can be found throughout the parenchyma as early as 3 hr post infarction and lasting up to 5 days.[5,92,93] Following ischaemic events, $CD4^+$ T-cell populations have two spikes in infiltration: following the first 3 hr and again after 24 hr.

Yilmaz *et al.*, provided a comprehensive picture of the important role played by T lymphocytes in cerebral ischaemia. Using T-cell-deficient mice, they evaluated infarct volume and neurological recovery following 60 min of tMCAO and observed reduced infarct size, indicating a novel role of T cells in brain injury and the neurological deficit seen with stroke.[94] In addition, Hurn *et al.* were able to recapitulate that data in 90-min tMCAO mice showing that both T and B cells have a role in the early damage within 24 hr of reperfusion.[95] In one of the first papers to inhibit T-cell migration to the brain during the acute phase of stroke, Liesz *et al.* administered very late antigen-4 and its counterpart vascular cell adhesion molecule-1 via monoclonal antibodies, which improved the outcome of stroke lesions by inhibiting lymphocyte invasion.[96] Moreover, recombinant T-cell receptor ligands and lymphocyte egress inhibitors such as fingolimoid have also been successful in murine models of stroke.[97–99]

Given the heterogeneity of T-cell phenotype, over the past 10 years researchers have been trying to elucidate the T-cell-specific factors responsible for contributing to stroke pathology. Shichita *et al.* showed that $\gamma\delta$ T cells, but not conventional $CD4^+$ T cells, can produce IL-17

following tMCAO, which contributes to ischaemic brain injury in an IL-23-dependent manner.[57] To clarify the mechanism for $\gamma\delta$ T-cell-produced-IL-17 contribution to stroke damage, Zhang *et al.* described that IL-17 causes reperfusion damage through the Calpain-transient reporter potential canonical (subtype) 6 pathway.[100] Following this, Arunachalam *et al.* clarified that the migration of these IL-17-producing cells to the stroke brain happens via CCR6 signalling as the ischaemic brains of CCR6-deficient mice are mostly devoid of IL-17.[101]

In their initial evaluation of lymphocyte influence to stroke damage, Yilmaz *et al.* also revealed the importance of IFN-γ, as splenocytes from IFN-γ-deficient mice did not confer the same levels of damage as splenocytes from wild-type mice when transferred into $Rag1^{-/-}$ mice following tMCAO.[94] More recently. Seifert *et al.* showed that administration of IFN-γ-neutralizing antibodies could block interferon-inducible protein 10 and subsequent brain damage and neurodegeneration following MCAO.[102] Another T-cell-derived cytokine that has been implicated in stroke damage is IL-21. We have shown that $CD4^+$ T-cell-derived IL-21 is a major contributor of reperfusion damage following tMCAO as $CD4^+$ T cells from IL-21-deficient mice do not cause the same levels of ischaemic damage compared with $CD4^+$ T cells from wild-type mice when transferred intravenously into T-cell-deficient mice.[103] Other cytokines such as IL-1β, TNF-α, and particularly IL-23 have also been shown to influence T cells following stroke indirectly.[104,105]

Recently, there has been a lot of excitement for the potential role of T regulatory (Treg) cells for protection against stroke damage. Liesz *et al.* showed that transfer of $Foxp3^+$ T cells into T-cell-deficient mice was protective and that Treg cells were present surrounding ischaemic brain areas at 7, 14 and 30 days following 30-min tMCAO.[106] Furthermore, Brea *et al.* administered a CD28 superagonist to target Treg proliferation, which reduced stroke-induced brain damage.[107] The migration of Treg cells during stroke pathology has been clarified by Li *et al.*, who suggest that CCR5-mediated recruitment of Treg cells is crucial to protect against blood–brain barrier disruption following stroke.[108] Lee *et al.* also suggested that CXCL14 can promote Treg differentiation and activation.[109] In contrast, Kleinshnitz *et al.* surprisingly showed that depletion of Treg cells using the DEREG mouse model reduces brain infarct size, suggesting that Treg cells can further brain damage by inducing BBB dysfunction.[110] Currently, the Treg cell contribution to ischaemic brain damage is still a highly debated topic.[111,112]

As lymphocytes are typically associated with adaptive immunity and antigen-specific immune responses, their dependency on specific antigens for contributing to stroke pathology has recently been evaluated. It was shown that adoptive transfer of myelin basic protein-tolerized splenocytes contribute to MCAO damage in rats.[113] This study concluded that immunological tolerance towards myelin basic protein could be transferred and induce neuroprotective effects potentially in a transforming growth factor-β_1-dependent fashion. In contrast, however, if rats are immunized with myelin basic protein and or peptides, they mainly induce T helper type 1 responses, resulting in worse stroke outcome.[114] The role of another myelin peptide-induced immunity, myelin oligodendrocyte glycoprotein $(MOG)_{35–55}$ has also been studied by several groups in the context of stroke. It was shown that mucosal induced tolerance by $MOG_{35–55}$ reduced infarct size by almost 70% following MCAO in an IL-10-dependent manner.[115] Conversely, Ren *et al.* demonstrated that MOG-specific splenocytes transferred into SCID mice can exacerbate infarct volume and associated neurological deficits, suggesting that antigen-specific T cells need to be tolerized to be beneficial.[116] More recently, Wang *et al.* evaluated the effect of administering DRα1-$MOG_{35–55}$, a compound that can inhibit neuroantigen-specific T cells and block the binding of chemokine-mediated migration. This study concluded that inhibiting $MOG_{35–55}$-specific T cells reduces brain damage following distal MCAO by shifting myeloid cells from a pro-inflammatory to an anti-inflammatory phenotype.[117]

The contribution to cytotoxic $CD8^+$ lymphocytes has also been assessed with regards to stroke pathology. In a clinical study, Schwab *et al.* identified the presence of $CD8^+$ T cells around infarcted lesions in patients with focal cerebral infarctions.[118] Furthermore, in aged mice inflicted with MCAO, a population of memory CD8 T cells were identified that contribute to injury by influencing microglia homeostasis.[16] $CD8^+$ T-cell-released factors have also been identified to contribute to stroke injury as cytotoxic protease granzyme-B has been identified to upregulate pro-apoptotic proteins in neurons following tMCAO[119] and is also present in human stroke lesions.[120] Furthermore, cytotoxic lymphocyte-produced perforin has also been identified to contribute to stroke damage in an antigen-dependent manner.[121] On the other hand, an anti-inflammatory population of IL-10-producing $CD8^+$ $CD122^+$ T cells was identified following MCAO in the ischaemic brain.[122]

Several groups have demonstrated that administration of B cells could be protective following stroke. Administration of B cells directly into striatum reduced infarct volume in tMCAO mice 48 hr after reperfusion.[123,124] The protective role of these B cells was later found to be dependent on IL-10 by the same group.[123] Furthermore, Monson *et al.* showed that generation of these protective B cells could be induced by hypoxic preconditioning and they are potentially recruited to the ischaemic brain by CXCL13.[125] Nonetheless, the role of B cells in contributing to stroke injury is somewhat controversial as their phenotype can be heterogeneous[126] and it has been

Table 1. Influence of T cells on the ischaemic brain in murine models of stroke

Cell type	Cytokine released	Mouse model	Effect on stroke volume	Reference
$\gamma\delta$ T cell	IL-17a	tMCAO	Increase	Shichita *et al.*[57]
CD4 T cell	IL-17a	tMCAO	Increase	Zhang *et al.*[18]
CD4 T cell	IL-17a	tMCAO	Increase	
Treg	N/A	tMCAO	Decrease	
Treg	IL-10	tMCAO	Decrease	Liesz *et al.*[106]
Treg	N/A	tMCAO	Increase	Kleinschnitz *et al.*[111]
CD4 T cell	IFN-γ	tMCAO	Increase	Yilmaz *et al.*[94]
CD4 T cell	IFN-γ	pMCAO	Increase	Seifert *et al.*[102]
CD4 T cell	IL-21	tMCAO	Increase	Clarkson *et al.*[103]
CD8 T cell	Granzyme B	tMCAO	Increase	Chaitanya *et al.*[119]
CD8 T cell	Perforin	pMCAO	Increase	Mracsko *et al.*[121]
B cell	IL-10	tMCAO	Decrease	Chen *et al.*[124]
B cell	N/A	tMCAO	Decrease	Monson *et al.* (2013)[125]
B cell	N/A	dMCAO	Increase	Doyle *et al.*[128]

dMCAO, distal middle cerebral artery occlusion; IFN-γ, interferon-γ; IL-17a, interleukin-17a; pMCAO, permanent middle cerebral artery occlusion; tMCAO, transient middle cerebral artery occlusion.

suggested that they have a minor, if any, role in contributing to ischaemic stroke during the acute phase.[127] Interestingly however, others have suggested that B cells have a role in mediating cognitive impairment in the weeks after stroke by producing harmful autoantibodies that inhibit long-term potentiation.[128] These studies highlight the diversity of lymphocyte contribution to ischaemic stroke pathology. The influence of lymphocytes on ischaemic pathology is summarized in Table 1.

Immune cells orchestrate dynamic changes to the neurovascular unit following ischaemic stroke

The cells that make up the neurovascular unit play a complex role in mediating BBB function, immune cell trafficking and injury after stroke[129–131] (Fig. 3). Several immune cells including neutrophils, DCs, lymphocytes and microglia, have been shown to directly mediate vascular injury during the acute phase of stroke to facilitate BBB dysfunction and consequent neuronal death. During both tMCAO and pMCAO, activated neutrophils can be seen within the leptomeninges as early as 6 hr, within the perivascular space by 15 hr, and within the CNS parenchyma by 24 hr.[132] IL-1 up-regulates the neutrophil-selective chemokines CXCL1 and CXCL2 within the plasma to recruit neutrophils into the brain during ischaemia.[133] In conjunction with this, intercellular adhesion molecule 1 has been shown to facilitate neutrophil adhesion to endothelial cells during MCAO,[134] and consequently to induce BBB dysfunction by disrupting the tight junction protein Claudin-5 through the production of MMP-9.[135–138] Inhibition of either neutrophil infiltration or neutrophil-derived MMP-9 reduces inflammatory-mediated cerebral damage and risk of haemorrhagic stroke,[135] suggesting that neutrophils can mediate BBB dysfunction and consequently affect neuronal health.

The role of infiltrating macrophages during ischaemic stroke is controversial, partly because both microglia and infiltrating macrophages were almost indistinguishable by cell surface markers as described above. CD11b-positive immune cells, which encompass both microglia and infiltrating macrophages, express abundant levels of angiopoietin-like protein 2 within the brain during tMCAO, which contributes to increased levels of the pro-inflammatory cytokines IL-1β and TNF-α to promote endothelial dysfunction and subsequent neurological deficits.[16,75] Additionally, $CD11b^+$ cells (expressed by both infiltrating macrophages and microglia) can signal through the platelet-derived factor receptor on arterioles to promote BBB permeability and the incidence of haemorrhage during stroke.[17] In contrast, depleting $CCR2^+$ monocyte infiltration increases BBB permeability and risk of haemorrhage after stroke, suggesting that infiltrating macrophages may play a role in maintaining brain vasculature.[77,139] In support of this, it has recently been shown that the myeloid cell and astrocyte-derived matricellular glycoprotein osteopontin is critically involved in astrocyte-mediated protection of the neurovascular unit after ischaemia; macrophage-specific depletion of matricellular glycoprotein osteopontin resulted in the failure of astrocytes to properly maintain cerebral blood vessels and BBB integrity after ischaemic stroke.[140] Macrophages are also hypothesized to promote vascular endothelial growth factor-mediated angiogenesis to promote neuroprotection and remove necrotic debris, termed the 'clean-up' hypothesis[141]; however, newly formed vessels are often leaky and it is unknown whether this is beneficial or detrimental following ischaemia.

As mentioned previously in this review, T cells play an intimate and complex role with the neurovascular unit during ischaemic stroke. Mechanistically, T cells have been shown to induce vascular dysfunction by promoting leucocyte/endothelial interactions through lymphocyte function-associated antigen 1/ intercellular adhesion molecule 1.[110] In this context, it is hypothesized that T cells play an important role in reducing neutrophil infiltration across the BBB, lymphocyte invasion, microglial activation and the production of pro-inflammatory cytokines through IL-10. It is still unclear how T-cell subtypes directly influence the neurovascular unit during cerebral ischaemia.

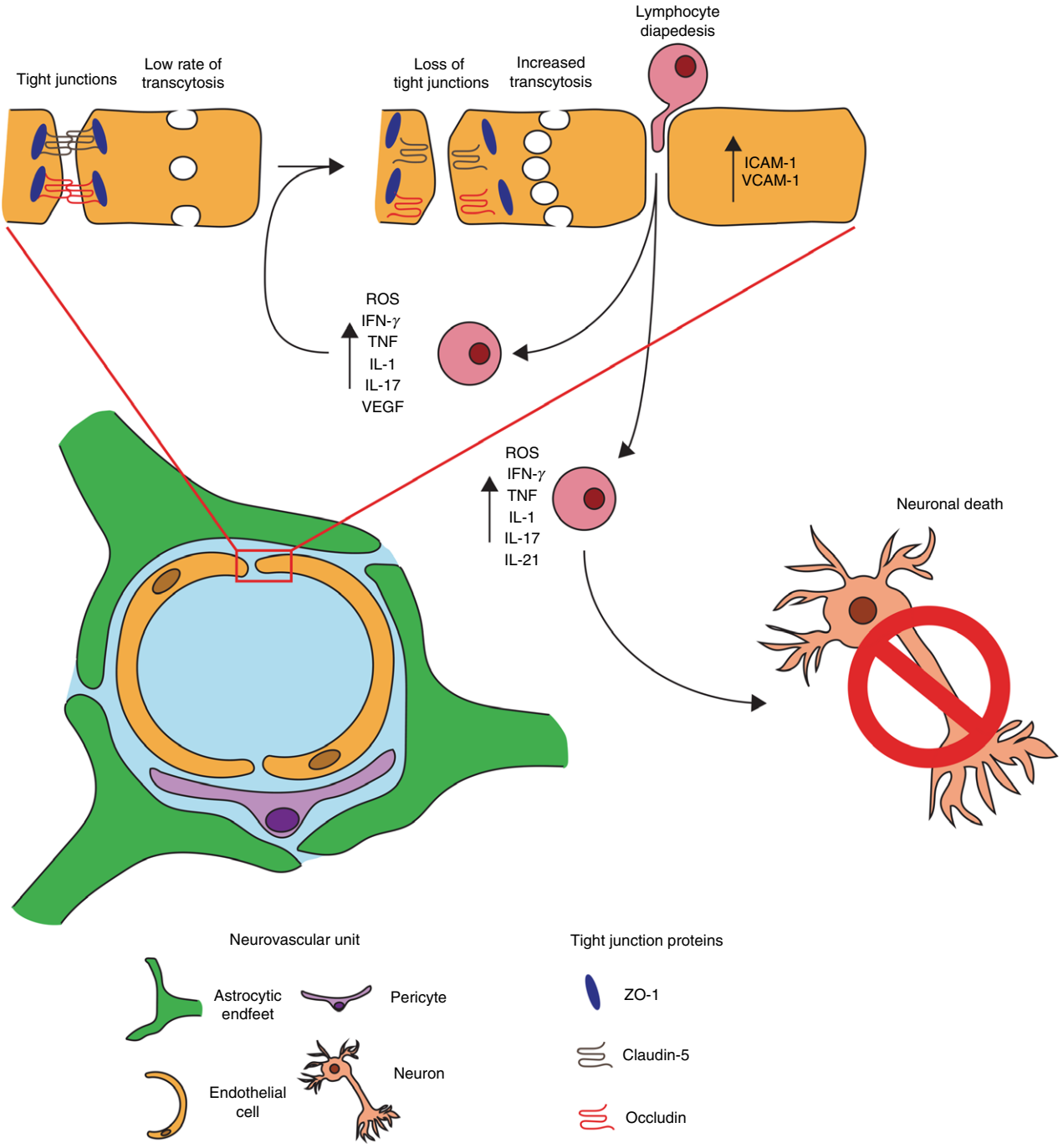

Figure 3. Schematic for how pro-inflammatory T cells impact the neurovascular unit (NVU) following brain ischaemia. Following brain ischaemia, damage to the endothelium results in the loss of tight junctions such as occludin, ZO-1 and Claudin-5. This process facilitates increased lymphocyte diapedesis through paracellular and transcellular pathways. Upon entry into the brain parenchyma T cells prorogate ischaemic damage by releasing pro-inflammatory factors and cytokines such as reactive oxygen species (ROS), interferon-γ (IFN-γ), tumour necrosis factor-α (TNFα), interleukin-1β (IL-1β), IL-17 and IL-21, which can act by further damaging cells of the NVU or by damaging neurons directly.

Conclusion

Many questions remain with regards to the roles and interactions of immune cells during stroke. Although there is a general consensus that in the acute phase of ischaemic stroke immune cells are primarily damaging, anti-inflammatory myeloid cells and lymphocytes can infiltrate the brain as well to influence functional recovery. It appears that the chronic phase after ischaemic stroke is a little more complex as lymphocytes can

develop tolerance, immunity and memory to various signals released from the ischaemic brain damage, which can determine how lymphocytes function. Nonetheless, recent experimental murine studies unveiling the molecular mechanisms of immune cell contribution to stroke have led to exciting findings that could be used at the clinic. Although these studies have been valuable and promising for future stroke therapies, an ongoing challenge will be the differences in gene changes across unbiased studies between humans and animal models that can arise due to heterogeneity between human samples, analyses of circulating factors in blood versus tissue parenchyma, and the timing of sample collection.

Overall, given the heterogeneous nature of immune cells and their influence on stroke pathology, it is essential to identify and target the specific cytokines that they produce to limit their detrimental effects and harness their benefits. Pursuing this will be critical to move forward and revolutionize the field. Apparent contradictions and current gaps in our knowledge of cellular immunity during stroke can now be further addressed in greater detail than ever before by using novel and emerging technologies such as single-cell RNA sequencing, cytometry by time-of-flight, two-photon imaging, whole-genome transcriptomic and epigenetic analysis with complementary bioinformatics, and unbiased proteomics among others. The use of these methods will not only serve to answer some the most vexing questions in the field, but will also lead to the discovery of novel pathways and a deeper understanding of the complex cellular interactions that occur during stroke. This will be critical in the development of new immunomodulatory therapies and novel treatments to mitigate, reverse and prevent the damage done during stroke.

Materials and methods

Mice

Wild-type C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME).

Transient middle cerebral artery occlusion

Focal cerebral ischaemia in mice was induced by occlusion of the left MCA, as described previously.[142] Operators performing surgeries were masked to experimental groups. In brief, the left common carotid artery was exposed, and the occipital artery branches of the external carotid artery (ECA) were isolated and coagulated. After coagulation of the superior thyroid artery, the ECA was dissected distally and coagulated along with the terminal lingual and maxillary artery branches. The internal carotid artery (ICA) was isolated, and the extracranial branch of the ICA was then dissected and ligated. A standardized polyamide resin glue-coated 6·0 nylon monofilament (3021910; Doccol Corp.,Sharon, MA) was introduced into the ECA lumen, and then advanced ~ 9–9·5 mm in the ICA lumen to block middle cerebral artery blood flow. During the entire procedure, mouse body temperature was kept between 37° and 38° with a heating pad. The suture was withdrawn 60 min after occlusion. The incision was closed, and the mice underwent recovery.

Lymphocyte isolation, intracellular cytokine staining and FACS

Mice were deeply anaesthetized with ketamine/xylazine and then transcardially perfused with cold phosphate-buffered saline (PBS). Single-cell suspensions were made from cervical lymph nodes and spleens by grinding the tissues between the frosted ends of glass slides. Red blood cells were lysed using ACK lysis buffer, and cells were washed with Hanks' balanced salt solution. Brains were minced with razor blades and pushed through 70-μm nylon cell strainers. Cells were washed, resuspended in 70% Percoll and overlaid with 30% Percoll. The gradient was centrifuged at 1200 rcf for 30 min at 4° without brake. The interface was removed and washed before plating. All collected organs were weighed, and live cells were counted using a haemocytometer.

Fluorescent microscopy

For frozen sections, mice were first perfused with cold PBS, followed by perfusion with 4% paraformaldehyde/PBS. Harvested tissues were left in 25% sucrose/PBS overnight at 4° and then embedded in Tissue-Tek OCT Compound (Sakura Finetek USA, Inc., Torrance, CA) before freezing at −80°. Five-micrometre-thick tissue cryosections were cut and stored at −80° until staining. Frozen sections were thawed for 10 min at room temperature and then placed in acetone for 10 min at −20°. Next, sections were incubated in PBS for 30 min at room temperature and then blocked with 2.4G2 antibody in 0·1% Triton X-100/PBS (1 : 50) for 30 min before applying anti-GFP-FITC primary antibody in 0·1% Triton X-100/PBS (1 : 100) for 1 hr at 37°. Sections were then washed three times for 10 min each time with PBS and mounted with ProLong Gold antifade reagent containing diamidino-phenyl indole (Invitrogen, Carlsbad, CA). All images were acquired with a camera (Optronics Inc., Goleta, CA) mounted on a fluorescence microscope (Olympus BX41, Leeds Precision Instruments). Individual fluorescent channel images were merged using PictureFrame software (Optronics Inc., Muskogee, OK). The brightness/contrast of the acquired digital images was applied equally across the entire image and equally to control images and analysed using Adobe Photoshop CS4 software (Adobe Systems Inc., San Jose, CA).

Antibodies

The following antibodies were purchased from BD Biosciences (Franklin Lakes, NJ): anti-CD4 Alx700 (RM4-5), anti-CD4 Alx647 (RM4-5), anti-CD8 PerCP (53-6.7), anti-B220 (RA3-6B2), anti-CD45 PerCP (20-F11); from eBioscience (San Diego, CA): anti-CD11b (M1/70); from Wako (Richmond, VA): anti-IBA-1 (019-19741); from EDM Millipore (Darmstadt, Germany): anti-GFAP (AB5541); from Millipore (Darmstadt, Germany): anti-NeuN (MAB377B). All secondary antibodies were purchased from Thermo Fisher Scientific (Waltham, MA).

Statistics

One-tailed unpaired Student's *t*-tests were computed using InStat software (GraphPad Software, La Jolla, CA) to make statistical comparisons between groups. Results are given as means plus or minus one standard deviation. Multiple comparisons were made using one-way analysis of variance. Where appropriate, two-sided Student's *t*-test analysis was used to compare measures made between two groups. *P*-values $< 0{\cdot}05$ were considered significant.

Ethics statement

All animal procedures used in this study were conducted in strict compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the University of Wisconsin Center for Health Sciences Research Animal Care Committee. All mice (~25 g) were anaesthetized with ketamine and xylazine for procedures, and all efforts were made to minimize pain and discomfort.

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Disclosures

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