UNIVERSITAT DE BARCELONA

Identity and functions of dendritic cell subsets in ischaemia-induced neuroinflammation

Mattia Gallizioli

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Identity and functions of dendritic cell subsets in ischaemia-induced neuroinflammation

Ph.D. thesis

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Barcelona, 2021

Identity and functions of dendritic cell subsets in ischaemia-induced neuroinflammation

Dissertation presented by **Mattia Gallizioli** in fulfilment of the requirements for the obtention of the doctoral degree of the University of Barcelona.

UNIVERSITY OF BARCELONA – FACULTY OF MEDICINE

DOCTORAL PROGRAMME IN BIOMEDICINE

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Abbreviations

AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic Acid
APC: Antigen-Presenting Cell
BATF: Basic Leucine Zipper ATF-Like Transcription Factor
BBB: Blood-Brain Barrier
BTLA: B- and T-lymphocyte attenuator
CCL: C-C motif Chemokine Ligand
CCR: C-C motif Chemokine Receptor
CD: Cluster of Differentiation
CDP: Common Dendritic Precursor
CNS: Central Nervous System
CSF: Cerebrospinal Fluid or Colony-Stimulating Factor
CXCL: C-X-C motif Chemokine Ligand
CXCR: C-X-C motif Chemokine Receptor
DALY: Disability-Adjusted Life Year
DAMP: Damage Associated Molecular Pattern
DC: Dendritic Cell
    cDC: conventional DC
    moDC: monocyte-derived DC
    pDC: plasmacytoid DC
DNGR: Dendritic Cell NK Lectin Group Receptor
DTX: Diphtheria Toxin
DWI: Diffusion-Weighted Imaging
EAE: Experimental Autoimmune Encephalomyelitis
eYFP: Enhanced Yellow Fluorescent Protein
FACS: Fluorescence Activated Cell Sorting
FLT: Fms-like Tyrosine Kinase
GFAP: Glial Fibrillary Acidic Protein
HIF: Hypoxia Inducible Factor
HMGB: High Mobility Group Box
ICAM: Intercellular Adhesion Molecule
IFN: Interferon
Ig: Immunoglobulin
IL: Interleukin
LFA: Leukocyte Function-Associated Antigen
Mac: Macrophage
MBP: Myelin Basic Protein
MHC: Major Histocompatibility Complex
Mincle: Macrophage Inducible $Ca2^{+}$-dependent Lectin Receptor
MMP: Matrix Metalloproteinase

MOG: Myelin Oligodendrocyte Glycoprotein
MRI: Magnetic Resonance Imaging
NET: Neutrophil Extracellular Trap
NMDA: N-Methyl-D-aspartic Acid
NO: Nitric Oxide
PRR: Pattern Recognition Receptor
PSGL: P-Selectin Glycoprotein Ligand
PWI: Perfusion-Weighted Imaging
r-tPA: Recombinant Tissue Plasminogen Activator
ROS: Reactive Oxygen Species
TCR: T Cell Receptor
TGF: Transforming Growth Factor
Th: Helper T cell
Treg: Regulatory T cell
TLR: Toll-like Receptor
tMCAO: transient Middle Cerebral Artery Occlusion
TNF: Tumour Necrosis Factor
VCAM: Vascular Cell Adhesion Molecule
VEGF: Vascular Endothelial Growth Factor

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Abstract

Cerebral ischaemia induces several inflammatory processes in the brain. Among them, the infiltration of immune cells is a hallmark of the pathology. Dendritic cells (DCs) are usually present in low numbers in the meninges and the choroid plexus, but rarely in the parenchyma. Upon ischaemia, the number of DCs increases, and the cells infiltrate the brain tissue, where they carry out different functions.

In an experimental murine model of stroke, we set out to investigate the infiltration of several subsets of DCs to the brain and their functional role. Early after stroke, we show a rapid and significant influx of DCs, especially of conventional type 2 DCs (cDC2), which are the most abundant subset at all time points analysed. Twenty-four hours after stroke, these cells were the major source of IL-23, which was able to stimulate its receptor on γδ T cells, inducing their production of IL-17. In turn, IL-17 is responsible for the stimulation of the production of *Cxcl1* by astrocytes, ultimately leading to the infiltration of neutrophils to the ischaemic brain and to the exacerbation of the tissue damage. We demonstrate that the interruption of the IL-23/IL-17 axis decreases the infarct size and improves the neurological outcome of stroke in mice, suggesting that cDC2 may play a detrimental role in the early phase of the immune response to stroke.

The analysis of the infiltration of DCs to the brain in inflammatory conditions has historically been difficult for the absence of univocal markers and for the similarity of their phenotype with other brain cells, especially microglia. The knowledge about the origin, phenotype and functions of brain DCs is therefore underdeveloped. One of the most commonly used markers for the study of DCs is CD11c, which is also expressed by a subset of microglia. The population of $CD11c^+$ cells present in the brain increases after stroke, and we show that $CD11c^+$ cells include proliferating microglia and infiltrating DCs. Despite their similarities, we demonstrate by RNA-Seq analysis that these two cell types exhibit a differential transcriptional profile, with interesting peculiarities in pattern recognition receptor and chemokine receptor expression. DCs extracted from the ischaemic brain outclass microglia in antigen presentation capacity, indicating a functional specialisation. We show that microglia are responsible for the production of chemokines that attract DCs to the brain, especially conventional type 1 DCs (cDC1). This specific subpopulation of DCs appears to have beneficial functions, reducing the infarct size and improving the functional outcome of ischaemic stroke.

Altogether, the studies presented in this thesis shed light on the features discriminating DCs from microglia and uncover previously unknown roles of diverse subpopulations of infiltrating DCs in the outcome of ischaemic stroke.

Introduction

The first goal after ischaemic stroke is to restore as quickly as possible the blood circulation to the ischaemic area. The survival of patients and the chances of good clinical outcome indeed largely depend on the effectiveness of reperfusion therapies. Apart from taking the flow-restoring vital interventions to a remarkable success rate, in the last few years research on stroke has been focusing on modulating the complex cascade of events that develop when the ischaemic tissue starts to suffer, to not only obtain reperfusion of the tissue, but also functional recovery.
Inflammation is one of the key elements in this pathological situation and its understanding and control is one of the broad objectives of this work, which faces the long-standing hurdle (Wiendl et al., 2015) of finding a successful brain-protective therapy for stroke. This dissertation is focused on the role that dendritic cells (DCs) may play in the context of cerebral ischaemia. Both the identification and definition of these cells characteristics in the inflamed brain were improved, and functions for specific DC subpopulations in driving the early post-stroke inflammation were uncovered.

In the following introduction I will resume the most important aspects of the scientific context in which this work was carried out.

1. STROKE

Stroke is a group of cerebrovascular pathologies that lead to the reduction of blood flow to a specific area of the brain. The two main types of stroke can be differentiated by their cause: ischaemic stroke is due to a blockage of a cerebral artery, whereas haemorrhagic stroke is due to a rupture. Their ultimate consequence is the lack of oxygen and nutrients to the tissue, leading to cell death.

Stroke is the second cause of death worldwide, accounting for approximately 11% of deaths, and is the second cause of Disability-Adjusted Life Years (DALY) loss.
Globally, over 13.7 million new strokes happen each year. According to current estimations, one in four people over age 25 will have a stroke during their lifetime (Johnson et al., 2019).
Although the mortality of stroke is slowly decreasing year by year, the burden of this disease is foreseen to increase because of the ageing of the population, which will predictably drive a surge in incidence (Truelsen et al., 2006).
Of all strokes, approximately 84% are ischaemic strokes. Of these, approximately 30% are lethal (Johnson et al., 2019). The rest of the cases leave the patients with a series of neurological deficits that impair their independence (Wolfe et al., 2011), leading to the aforementioned loss of DALYs.

This dissertation and the underlying project focus on ischaemic stroke, with few mentions of haemorrhagic stroke.

The most effective strategy to combat stroke today is prevention, since all modifiable risk factors combined account for about 88% of the global stroke burden, as measured by DALYs lost (Lindsay et al., 2019). When prevention is not enough and a stroke does happen, the objective of the currently available interventions is to restore the blood flow to the ischaemic area as rapidly as possible, to try to rescue the tissue that has not died between the moments of occlusion and reperfusion. To date, the available approaches to restore perfusion of the brain tissue are the administration of recombinant tissue plasminogen activator (r-tPA), mechanical thrombectomy and their combination (Prabhakaran et al., 2015). The critical feature of both these therapies is that their efficacy largely depends on the onset-to-treatment time (up to 6h after stroke onset for r-tPA, up to 24h for mechanical thrombectomy in the most favourable cases) (Prabhakaran et al., 2015). Remarkable progress has been made, and huge public health efforts are in place to allow most patients to receive proper treatment in this time window, nonetheless contraindications exist for both therapies and not always a complete reperfusion is ultimately achievable (Prabhakaran et al., 2015).

Hence, brain-protective strategies in combination with reperfusion therapies are under active investigation for the treatment of stroke and are aimed at avoiding further damage to the tissue apart from the one directly resulting from ischaemic cell death (Chamorro et al., 2016). The objective of these strategies is to control the detrimental effects of the cascade of pathological events that develops at the onset of stroke and during the subsequent minutes, hours, days, even when reperfusion is achieved.

1.1. ANIMAL MODELS OF CEREBRAL ISCHAEMIA

The vast majority of knowledge available about the pathophysiology of stroke is derived from animal studies (Dirnagl et al., 1999). Several animal models of brain ischaemia have been established, and rodents are the most commonly used for several, mostly practical, reasons (Hossmann, 2008). The models that best reproduce the clinical condition of ischaemic stroke are the ones where a focal ischaemia is produced, whether by mechanical occlusion or by the induction of thrombus formation (Carmichael, 2005). Since the most frequently affected vascular territory in human ischaemic stroke is the one irrigated by the middle cerebral artery (MCA) (Howells et al., 2010), and given the relative similarity of the

rodent cerebral vasculature (Lee, 1995), several models aim at producing ischaemia by occluding the MCA (Bacigaluppi et al., 2010).

The model we used for the studies presented here is the transient intraluminal MCA occlusion (tMCAO), which is achieved by inserting a nylon filament with a silicone tip through an access in the external carotid artery and leading it via the internal carotid artery to occlude the origin of the MCA (Longa et al., 1989) **(Figure 1)**. After 45 minutes, the filament is retracted to allow reperfusion. The technical details of the surgery are reported in the methods sections of the articles presented.
This surgical approach allows to produce a focal ischaemia and to study the pathological mechanisms that develop during the occlusion and after reperfusion, thus modelling the situation in which a stroke patient achieved a successful recanalisation of the MCA.

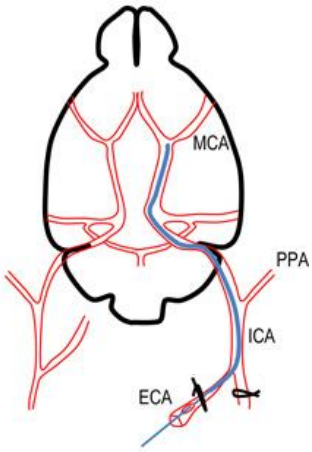

Figure 1. Transient middle cerebral artery occlusion: schematic representation.
The filament is inserted through an opening in the external carotid artery (ECA) and pushed through the internal carotid artery (ICA) to reach and occlude the origin of the MCA. PPA; pterygopalatine artery. Adapted from (Rousselet et al., 2012).

It is of course important to keep in mind that models have limitations. The tMCAO model is one of the least invasive surgical models of stroke available; nevertheless, a surgical manipulation, with the relative anaesthesia and analgesia, is necessary. Furthermore, in the interpretation of the results we must consider the peculiarities of the model used when compared with humans, from the differences in the brain tissue (for the mouse: lissencephaly, low white matter proportion) to the ones observed in the immune compartment (Mestas and Hughes, 2004).

1.2. PATHOPHYSIOLOGY

Neurons have a high demand for oxygen and glucose delivery since they are not able to efficiently store energy. This makes the brain especially sensitive to ischaemia (Mergenthaler et al., 2013).
The occlusion of a brain artery generates a gradient of focal hypoperfusion that leads to the formation of an ischaemic core, where the tissue irreversibly dies by necrosis due to severe metabolic failure and consequent breakdown of ion homeostasis (Brouns and De Deyn, 2009).

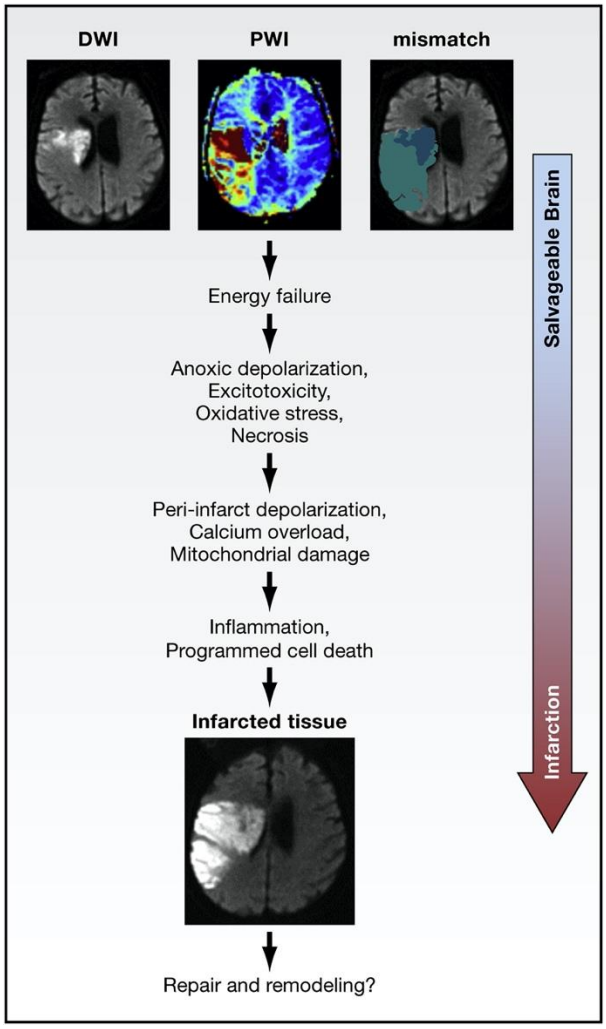

Figure 2. From hypoperfusion to infarct: the penumbra.
The penumbra is defined by magnetic resonance imaging (MRI) through the concept of mismatch, i.e. the difference between alteration in Diffusion Weighted Imaging (DWI), indicative of tissue damage, and the alteration in Perfusion Weighted Imaging (PWI), which measures cerebral blood flow. The penumbra is the region where the tissue is still viable, but it is functionally impaired due to lack of blood supply. This tissue is potentially salvageable if the underlying pathological processes are managed. Source: (Moskowitz et al., 2010).

Thanks to the presence of variably abundant collateral circulation, a region of functionally impaired but viable tissue, the penumbra, surrounds the core. Its condition can evolve favourably – or not – depending on rapid reperfusion and swift control of the underlying pathological processes (Moskowitz et al., 2010) **(Figure 2)**.

In a complex series of events, whose detailed molecular mechanisms go beyond the scope of this introduction, the dysregulated depolarisation of stressed neurons induces accumulation of extracellular glutamate, which in turn activates α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and N-methyl-D-aspartate (NMDA) receptors on neighbouring neurons, generating a spreading excitatory feedback loop that leads to excitotoxicity.
Overstimulation of these receptors can induce further downstream cellular effects including abnormal influx of calcium ions, induction of free radicals production, mitochondrial damage and ultimately DNA damage and cell death, by necrosis or apoptosis.

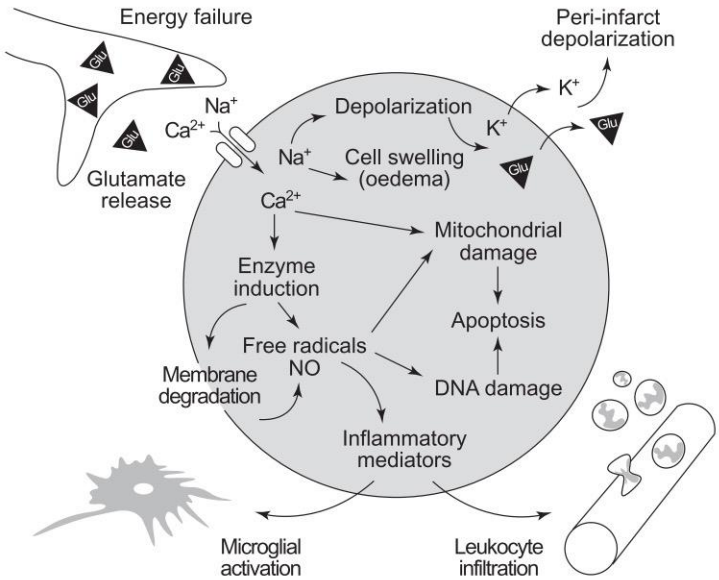

Figure 3. Pathophysiological mechanisms in the acute phase of brain ischaemia.
A complex series of extra- and intra-cellular events develop after cerebral ischaemia. Source: (Dirnagl et al., 1999).

Necrotic cell death provokes a robust inflammatory response in the brain, with complex consequences that can at first exacerbate the lesion, but also lay the foundation for the subsequent tissue repair (Dirnagl et al., 1999; Moskowitz et al., 2010) **(Figure 3)**.

One of the first inflammatory stimuli comes from the intravascular compartment, where the occlusion of the vessels and the consequent flow stagnation leads to production of reactive oxygen species and to the activation of the coagulation and complement cascades (Peerschke et al., 2010).
Adhesion molecules are readily exposed on the surface of platelets and endothelial cells, facilitating the tethering and rolling of leukocytes (Yilmaz and Granger, 2010). The blood-brain barrier (BBB) is a complex structure composed of endothelial cells, pericytes, astrocytic endfeet and extracellular matrix, which is the main gatekeeper responsible for the fine control of the physical separation between the brain parenchyma and the peripheral immune system. Oxidative stress and inflammatory signals can alter its permeability through the activation of matrix metalloproteinases, which are able to digest the endothelial basal lamina (Gidday et al., 2005). Apart from allowing the extravasation of high molecular weight molecules, which drives vasogenic oedema, the leakage from the BBB is a key facilitator of the migration of blood-borne cells to the brain parenchyma (del Zoppo, 2009). Signals released by the dying tissue also contribute to the inflammatory response of the brain: mainly products derived from the pathways leading to oxidative stress, apoptosis and necrosis – the so-called damage-associated molecular patterns, DAMPs (Bianchi, 2007).

The following chapters represent a deeper dive into these complex inflammatory mechanisms, which set the stage for the involvement of DCs in the immune response to stroke.

1.2.1. NEUROINFLAMMATION

Already before any cell death occurs, the release of ATP into the extracellular space, mainly by stressed depolarising neurons, induces activation of microglia by the engagement of the pattern recognition receptor (PRR) P2X7 (Iadecola and Anrather, 2011) **(Figure 4)**.

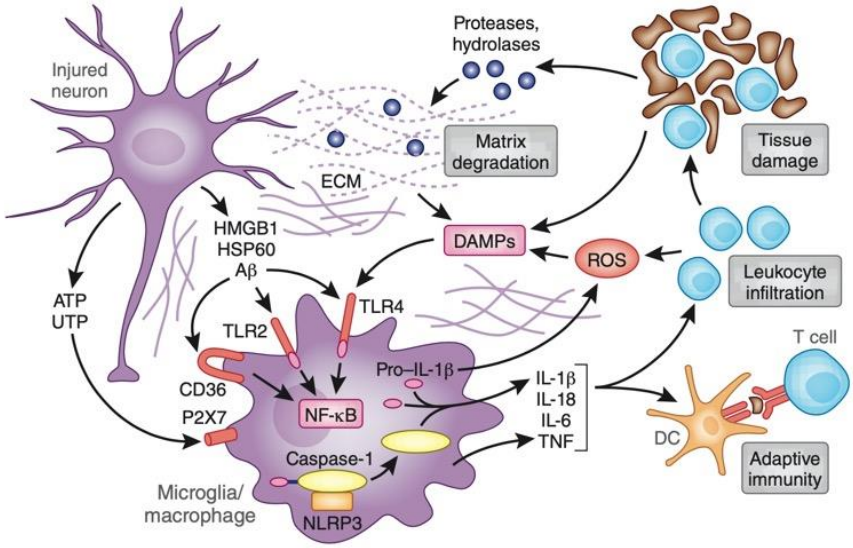

Figure 4. DAMPs activate microglia.
The release of DAMPs and nucleotides stimulates PRRs on microglia, leading to the production of pro-inflammatory cytokines and reactive oxygen species, which contribute to the inflammatory milieu of the ischaemic brain. Source: (Iadecola and Anrather, 2011).

In parallel, the rise in extracellular glutamate activates metabotropic glutamate receptors, inducing a pro-inflammatory phenotype (Chapman et al., 2000).
When cells begin to die, the release of DAMPs, such as high mobility group box 1 (HMGB1), heat shock proteins, peroxiredoxin and purines, becomes the main source of activation for microglia, perivascular macrophages and endothelial cells of the brain (Gülke et al., 2018).

Loss of cell-to-cell contact between dying neurons and microglia is also an activation signal for the latter, because of the disruption of two main regulatory axes like CD200-CD220R and CX3CL1-CX3CR1 (Cardona et al., 2006; Hoek, 2000).
All these pathways converge on stimulating the production of pro-inflammatory cytokines (TNFα, IL-6, IL-1β), nitric oxide (NO) and matrix metalloproteinases (MMPs) by microglia and resident macrophages (Gülke et al., 2018).
The initial activation of microglia and resident cells, and their polarisation towards an inflammatory phenotype is a physiological reaction to the alteration of cerebral homeostasis, possibly aimed at the containment of the lesion by phagocytosing cell debris and the first infiltrating leukocytes (Otxoa-de-Amezaga et al., 2019b).
Astrocytes are involved in this acute build-up of inflammatory products too. They are sensitive to the absence of nutrients due to ischaemia, leading to a reduced capacity of glutamate uptake, contributing to excitotoxicity (Bylicky et al., 2018),

loss of neurotrophic function and to impairment of their coverage of the blood-brain barrier (Jayaraj et al., 2019).
Furthermore, they can also directly contribute to the inflammatory milieu by releasing cytokines and metalloproteinases in response to DAMPs, although with extremely heterogeneous responses (Zamanian et al., 2012).

Altogether, the by-products of this early activation of resident cells end up resulting deleterious in the sub-acute phase of stroke, since they further destabilise the blood-brain barrier. Early after ischaemia there is a reduction of almost all the main components of the tight junctions between endothelial cells, such as occludin and zonula occludens-1 proteins, VE-cadherin and claudin 5. Furthermore, endothelial cells of the post-capillary segments can swell and detach from the basal lamina, further aggravating the cycle of hypoxia and hypoperfusion because of secondary microvascular occlusions (Petrovic-Djergovic et al., 2016). MMPs degrade the extracellular matrix, disrupting the scaffold that supports the outer layer of the barrier and that allows pericytes and astrocytic endfeet to stay in place. Transient activation of MMP2 in the initial phase, followed by induction of MMP3 and MMP9 expression and activation are key contributors to the post-ischemic BBB breakdown. (Abdullahi et al., 2018; Yang and Rosenberg, 2011) **(Figure 5)**.

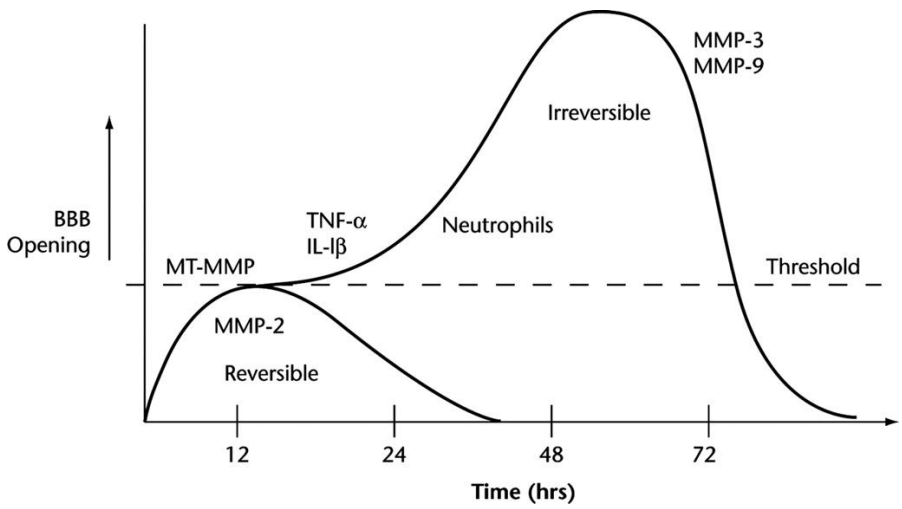

Figure 5. Schematic representation of BBB injury upon brain ischaemia.
The opening of the blood-brain barrier appears to be biphasic and caused by different mechanisms that can lead to reversible or irreversible damage. Source: (Yang and Rosenberg, 2011).

In a simplified summarisation, the end result in the acute phase is an increased paracellular leakage of fluids, solutes and blood-borne cells into the brain parenchyma.

During the first stages of ischaemia, various chemokines responsible for the migration of resident cells towards the lesion and for the attraction of peripheral cells to the brain are rapidly expressed. This initial production is mainly attributed to activated microglia, but also astrocytes and neurons participate in the accumulation of chemoattractant factors in the brain parenchyma and microvasculature (Chen et al., 2018) (partially resumed in **Table 1**).

Table 1. Role of chemokines in ischaemic stroke.
Table adapted from (Chen et al., 2018).

Chemokine	Receptor	Role in inflammation	Role in neuroprotection
CCL2	CCR2	Upregulation in damaged brain (rat) Promotion of infiltration in CNS (mouse) Reduction of infarct size in knockout mice (mouse) Reduction of BBB integrity (in vitro, mouse)	Promotion bone marrow stem cell and neuroblast migration (rat) Participation in hypoxic preconditioning protection (mouse)
CCL3	CCR1/5	Upregulation in ischaemic brain (rat) Recruitment of neutrophils (mouse)	Unknown
CCL5	CCR1/3/5	Upregulation in stroke (human) BBB breakdown (mouse)	Neuronal protection (human, mouse)
CCL11	CCR3	Unknown	Promotion of neural precursor cell (NPC) migration (mouse)
CXCL1	CXCR2	Upregulation post-stroke (mouse)	Angiogenic activity (monkey)
CXCL2	CXCR2	Upregulation post-stroke (mouse)	Unknown
CXCL5	CXCR2	Upregulation post-stroke (human)	Unknown
CXCL8	CXCR1/2	Upregulation post-stroke (rabbit) Recruitment of polymorphonucleate cells (human)	Promotion of mesenchymal stem cell (MSC) migration (rat) Enhancement of angiogenic potential of MSC (rat)
CXCL10	CXCR3	Upregulation of post-mortem (human) BBB breakdown (human, mouse)	Unknown
CXCL12	CXCR4/7	Upregulation in ischaemic penumbra (mouse) Promotion of leukocytes infiltration (mouse)	Promotion of homing of NPCs (rat), hematopoietic stem cells (mouse) Promotion remyelination (mouse)
CXCL16	CXCR6	Upregulation in stroke (human)	Recruitment of glial precursor cells (in vitro, mouse)
CX3CL1	CX3CR1	Upregulation post-stroke (rat) Enhancement of inflammation (mouse)	Reduction neuronal apoptosis (in vitro, mouse) Induction of neovascularization (rat) Promotion of stem cell migration (rat)

In parallel, the expression of adhesion molecules on the luminal side of endothelial cells is also implicated in the infiltration of peripheral cells. E- and P-selectins are mainly responsible for the attachment of neutrophils, monocytes and lymphocytes to the vessel walls and to platelets respectively, priming their activation and extravasation (Malone et al., 2019).

Several components of the cell adhesion molecule (CAM) family also show increased level of expression on the endothelial layer after stroke. Among these, intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) were strongly implicated in stroke pathophysiology (Yilmaz and Granger, 2010).
CAMs interact with integrins expressed on the surface of leukocytes, which are also overexpressed in inflammatory conditions. Among these, lymphocyte function-associated antigen 1 (LFA-1), macrophage antigen 1 (Mac-1) and integrin β2 (CD18) are the ones that most reinforce the adherence of circulating leukocytes to the vessel wall (Yilmaz and Granger, 2010).

1.3. THE INVOLVEMENT OF IMMUNE CELLS

It should by now be evident how the relationship between the ischaemic brain tissue and the immune system depends on an interplay of signals coming from resident cells, which readily respond to the damage, and immune cells recruited to the site of the lesion. The first peripheral responders in turn bring in further inflammatory cues that magnify the reaction, but can also drive it towards resolution.

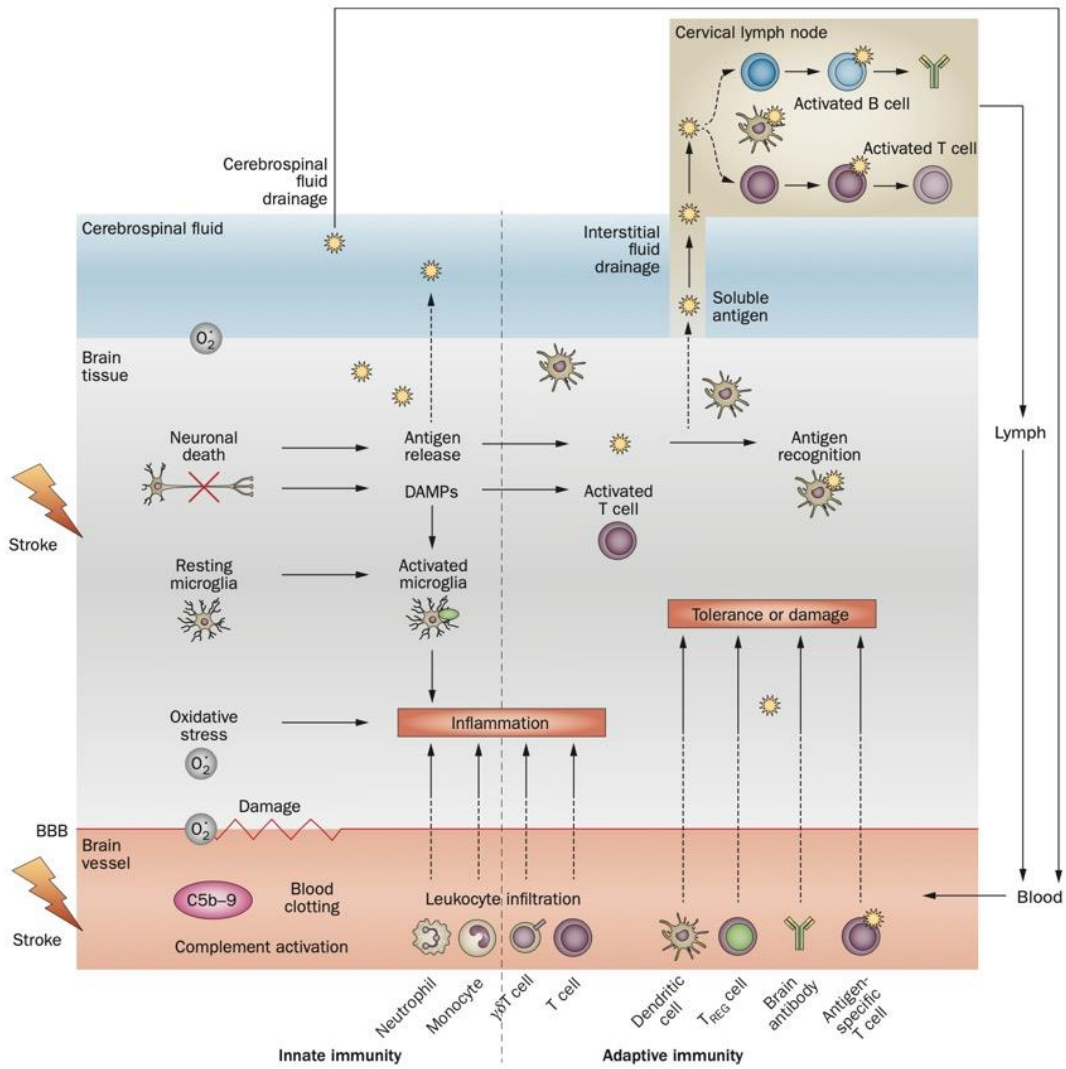

Figure 6. Cascade of events following brain ischaemia.
A strictly regulated cascade of events happens after stroke, with DAMP release and oxidative stress as main molecular initiators and propagators of inflammatory cues. The activation of microglia and the infiltration of peripheral immune cells are crucial steps in the evolution of the lesion, both for the initial inflammation and the subsequent tissue repair. Central nervous system (CNS) antigens can be released into the system through various routes and can activate adaptive immune responses. Source: (Chamorro et al., 2012).

The strictly regulated activity of immune cells, both of the innate and adaptive arms, in the healthy brain is what establishes the contemporary definition of immune privilege, which is not just a passive condition of isolation of the brain parenchyma due to the presence of physical barriers (Galea et al., 2007). The immune reaction that takes place after a stroke represents an acute alteration of this condition that can lead to far-reaching and long-lasting consequences (Chamorro et al., 2012) **(Figure 6)**.

In a seminal work for the field, Gelderblom et al. studied the infiltration dynamics of many of the different immune cell populations that get involved after experimental stroke in mice, resuming it in a graph that also hints at their reciprocal relationships (Gelderblom et al., 2009) **(Figure 7)**. More recent studies anticipated the entrance of neutrophils as early as 3h after stroke, together with monocytes and macrophages, all peaking between 2-3 days after stroke (Chu et al., 2014).

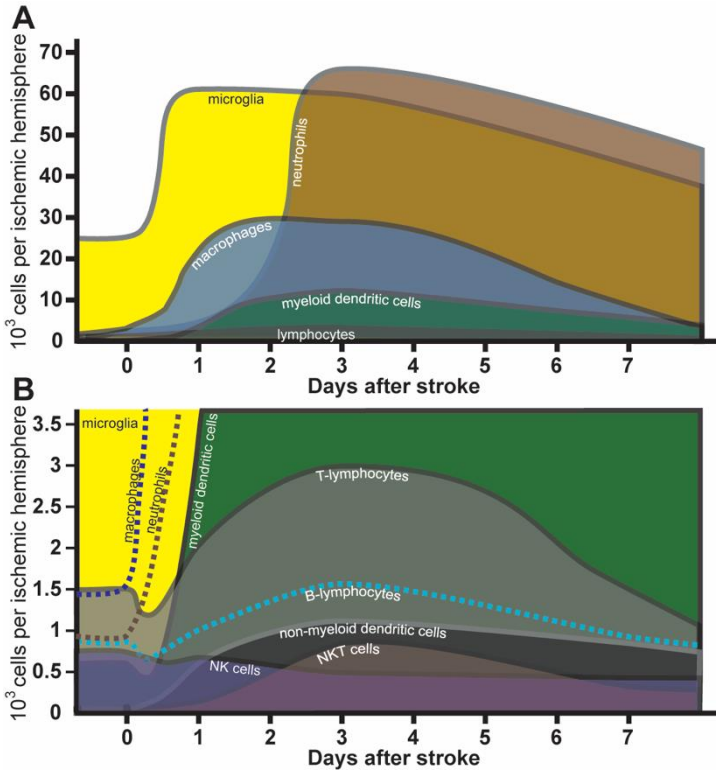

Figure 7. Temporal dynamics of post-stroke immune cell infiltration.
(A) The graph represents the number of cells per hemisphere in the lesioned side of the mouse brain after transient middle cerebral artery occlusion (tMCAO). The curves are extrapolated from data obtained from sham animals (before 0) and from mice subjected to tMCAO, analysed by flow cytometry 12h, 1, 3 and 7 days after reperfusion. (B) Magnification of lower portion of graph in A. Source: (Gelderblom et al., 2009).

The following paragraphs are an introduction of the main players of the post-ischaemic immune reaction, leaving a dedicated chapter for dendritic cells, the main topic of this dissertation.

1.3.1. MICROGLIA

The first responders of the innate branch of immunity are microglial cells. For their number and ubiquitous localisation in the brain they are poised to maintain homeostasis and react in case of alterations. They are the first line of defence in case of pathogen invasion and their phagocytic activity in adulthood is responsible for the clearing of dying cells and cellular debris, and for synaptic pruning (Li and Barres, 2018).

Microglia are generated early in development by precursors that colonise the brain at embryonic stages. They migrate from the yolk sac and develop in the brain in PU.1- and TGF-β-dependent way, to differentiate themselves from other macrophages already at E16.5. Their exact ontogeny is however to date under very active investigation (Thion et al., 2018; Utz et al., 2020).

Adult microglia are relatively long-lived and their slow turnover is maintained through self-replication, largely dependent on colony stimulating factor 1 receptor (CSF1R) signalling (Elmore et al., 2014).

Microglia present a unique transcriptomic signature when compared with other central nervous system (CNS) monocyte/macrophage cells (Butovsky et al., 2014), with some key genes identified as specific of adult murine microglia, such as *Sall1*, *Hexb*, *Tmem119*, *P2ry12* and *Trem2* (Hammond et al., 2019; Li and Barres, 2018).

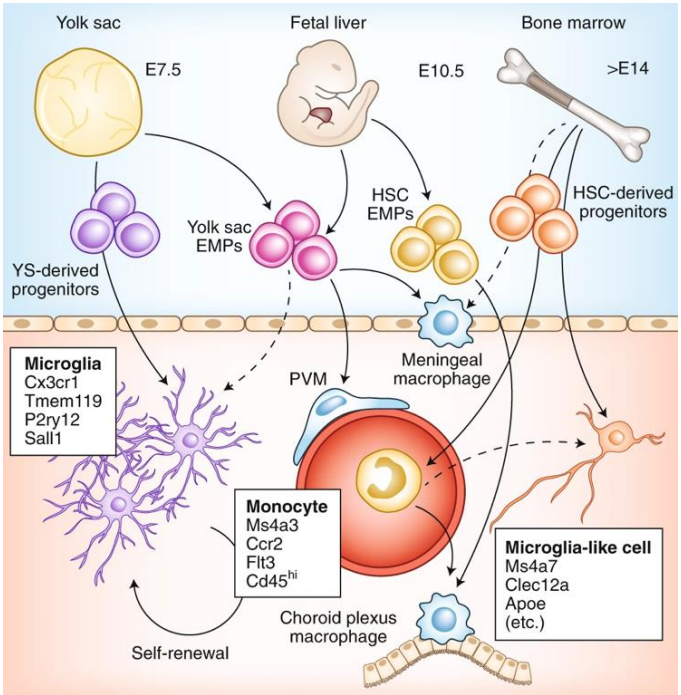

Figure 8. Origin of microglia and microglia-like cells.
Microglia derives from yolk sac progenitors that enter the brain in embryonic stages, differentiate and then self-renew by proliferating. Other macrophages can derive from hematopoietic progenitors, while monocytes can enter via the blood stream. Source: (Bennett and Bennett, 2020).

The definition of a unique and easily recognisable phenotype has been difficult, since the majority of superficial markers are shared among other CNS and peripheral infiltrating macrophages, but the expression of the transmembrane proteins TMEM119 and CX3CR1, and of the purinergic receptor P2RY12 have been recently proposed as good tools to study microglia (Bennett et al., 2016) **(Figure 8)**.

The morphology of microglia is not especially recognisable either, since again it is shared with other macrophages and carries an added layer of complexity that resides in its extreme plasticity. In steady-state conditions microglial cells present small cell bodies and large ramifications, responsible for scanning the surrounding microenvironment, much like dendritic cells. When activated, they rapidly pass through a spectrum of morphological changes that leaves them with a more amoeboid form, most adequate for phagocytic activity (Fumagalli et al., 2019; Otxoa-de-Amezaga et al., 2019b).
To complicate matters, the heterogeneity of the microglial population has clearly emerged in the last few years, mainly thanks to new techniques such as single-cell transcriptome analysis or time-of-flight mass cytometry (CyTOF). During the development of the brain, from embryonic stages to adulthood and elder life, the transcriptome of microglia has been shown to change, together with its functions (Silvin and Ginhoux, 2018). Similarly, a complex spatial heterogeneity both in health and disease has been very recently unravelled (Hammond et al., 2019; Masuda et al., 2019; Mrdjen et al., 2018) **(Figure 9)**.

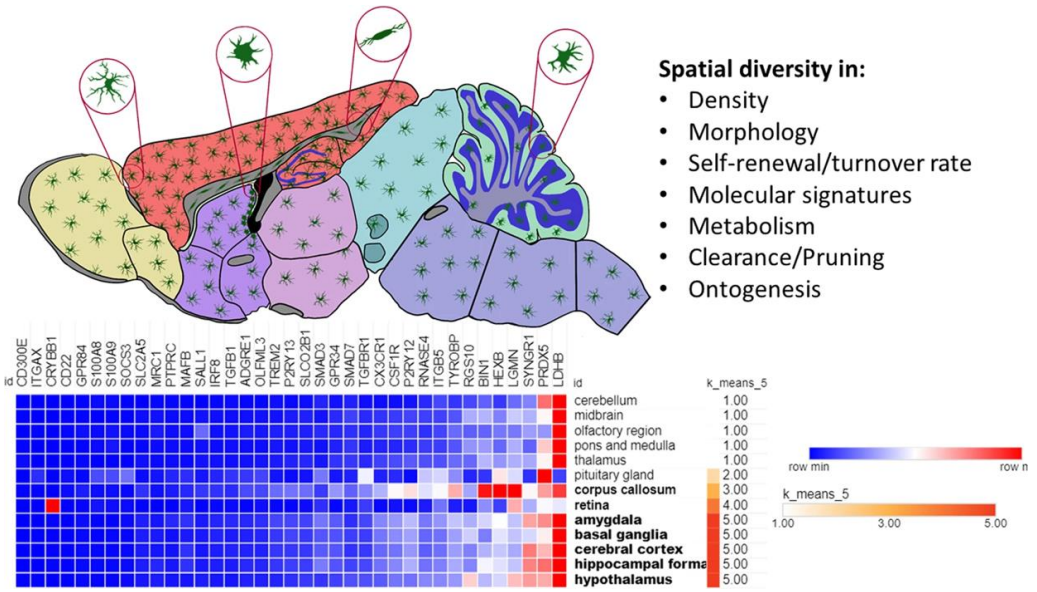

Figure 9. Regional heterogeneity of microglia in the brain.
Differences in cell number, gene expression and functions have been observed throughout different regions of the brain. Temporal heterogeneity is an added layer to be considered. The heatmap represents RNA-Seq data of microglial genes from various brain regions. Source: (Tan et al., 2020).

Furthermore, it is important to note that this heterogeneity is not at all static, or only dependent on the ontogeny of the subsets, but it is constantly changing in concert with the local environment (Bennett et al., 2018).
The activation of microglia and the morphological changes that concur make the ischaemic brain tissue a particularly challenging scenario for the differentiation of these cells from resident or infiltrating macrophages, especially with the techniques most commonly available.
The main differentiating factor regarding the response to ischaemia is possibly the timing: microglial cells are the first to activate and respond to alterations of the homeostasis. The initial response of microglia is characterised by the increased expression of activation markers such as class II major histocompatibility complex (MHCII), sometimes accompanied by co-stimulatory molecules CD80 and CD86, CD40 and ICAM (Lynch, 2009), and by the production of pro-inflammatory cytokines such as TNF-α IL-1β and IL-6, which participate in the early aggravation of the neuroinflammatory milieu of the ischaemic brain. Microglia are also an early source of chemokines responsible for the infiltration of peripheral cells such as CCL2, 3, 4, 5 and others (Aloisi, 2001).
The phagocytic activity that starts soon after (Schilling et al., 2005) is possibly a signal that – again, in an heterogeneous time- and space-dependent fashion – some microglial cells are already in early phases transitioning to a resolutory phenotype (Ransohoff, 2016). In this sense, our group has recently demonstrated that microglial phagocytosis constitutes an important mechanism to regulate the amount of neutrophils in the ischaemic parenchyma and consequently control the evolution of the infarct lesion (Otxoa-de-Amezaga et al., 2019b).
The switch to a phagocytic phenotype can also drive the production of anti-inflammatory mediators such as IL-10 and TGF-β (Nathan and Ding, 2010), which can be immunoregulatory in later phases, especially when lymphocytes get involved (Iadecola and Anrather, 2011).
Nevertheless, this phagocytic activity could also be the prelude to a possible role of microglia as antigen-presenting cells, which has been proposed in different pathological settings (Shaked et al., 2004; Wlodarczyk et al., 2014). The consequence of this putative interaction between microglia and T cells is however still unclear since early studies showed the induction of anergy, whereas later studies the activation of regulatory T cells, even though the overall outcome appears to be predominantly neuroprotective (Schetters et al., 2018).

For the scope of this work it is important to note that several specific genetic and pharmacological tools have been developed both to track and to deplete microglia, some of which have been very useful to us (Bennett et al., 2016). For the tracking

and genetic identification of microglia we used the Cx3cr1^{CreERT2} mice crossed with ROSA26-tdTomato mice, obtaining specific expression of the tdTomato fluorescent protein in cells that express the Cre recombinase under the control of a tamoxifen-inducible *Cx3cr1* promoter (Yona et al., 2013).
As for the pharmacological tools, we took advantage of the dependence of microglia on the CSF1 signalling pathway, by interfering with it. We administered to mice the drug PLX5622, an inhibitor of the CSF1 receptor, which is known to deplete almost completely the population of microglia (Elmore et al., 2014). Further details can be found in the methods section of the second article presented in this dissertation.
All in all, it is evident that the position and reactivity of microglia make them the perfect early sensors and first line responders to the alteration of homeostasis that develops soon after cerebral ischaemia and involve them both in the initial inflammatory reaction and in the subsequent path towards resolution.

1.3.2. NEUTROPHILS

Neutrophils are among the first peripheral cells to invade the brain after ischaemia (Chu et al., 2014; Gelderblom et al., 2009). The involvement of these cells in the outcome of ischaemia is still controversial, possibly due to the contemporary presence of different subsets, but the majority of actions that they carry out in the setting of cerebral ischaemia points at them as mainly pro-inflammatory mediators and harmful players (Jickling et al., 2015). Overall, it has been demonstrated long ago that the degree of infiltration correlates with stroke severity in patients (Akopov et al., 1996).
Neutrophils adhere to the microvasculature of the brain as soon as 2 hours after ischaemia, due to the increased expression of adhesion molecules on the damaged endothelium (see chapter 1.2.1) and to upregulation of adhesion receptors on their own surface, with a relevant role played by P-selectin glycoprotein ligand 1 (PSGL-1)-mediated platelet interaction (Sreeramkumar et al., 2014). This accumulation in the microvasculature could contribute to secondary micro-thrombosis through interaction with platelets, release of neutrophil extracellular traps (NETs) and local activation of the coagulation cascade (Jickling et al., 2015).

Various chemokine pathways have been implicated in the attraction of neutrophils to the site of the ischaemic lesion (Jickling et al., 2015), some of which are more relevant than others to this dissertation: CCR1, CCR2 and CCR5 knockout mice showed reduced infiltration (Reichel et al., 2006); CXCL-1, -2, -5 and -8 chemokines released from parenchymal cells activate and recruit neutrophils, a classical pathway of inflammatory states (Kolaczkowska and Kubes, 2013) **(Figure 10)**.

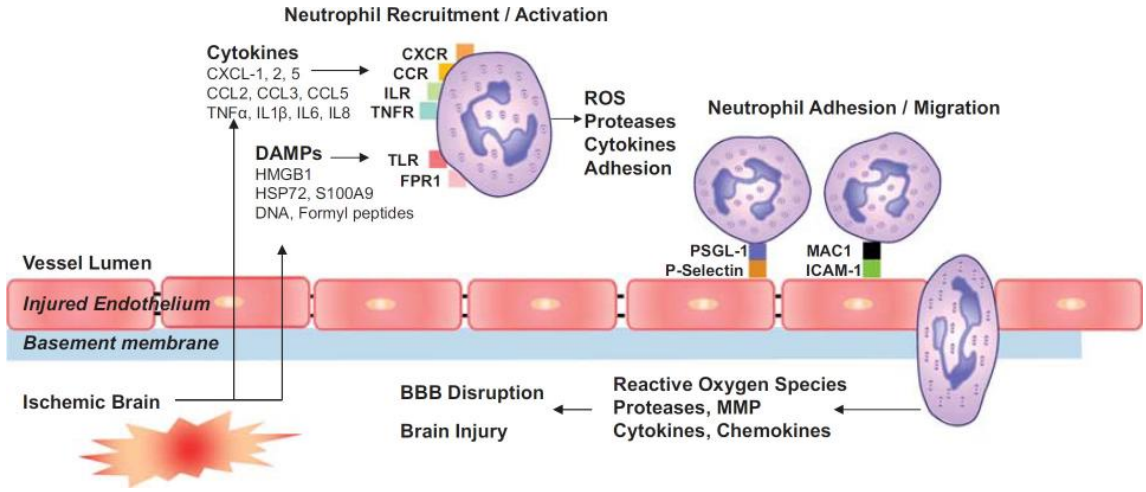

Figure 10. Neutrophil activation and adhesion in acute ischaemic stroke.
Neutrophils adhere to the endothelium and migrate into the parenchyma, with deleterious effects on both the blood-brain barrier and the brain tissue. Source: (Jickling et al., 2015).

The release of various proteases by neutrophils contributes to the disruption of the blood-brain barrier, possibly even leading to haemorrhagic transformation (Fernandez-Cadenas et al., 2013). Among these, MMP9 and elastase appear to be the most deleterious (Rosell et al., 2008; Stowe et al., 2009).
Neutrophils are also a prominent source of reactive oxygen species that contribute to the local early neuroinflammation (Matsubara et al., 2007).
Moreover, they have been implicated as mediators in the worsening of ischaemic stroke outcome after systemic inflammation stimuli via an IL-1-dependent mechanism (McColl et al., 2007).
Though the evidence for an overall detrimental role of neutrophils in the context of cerebral ischaemia is abundant and strong, there are also hypotheses and (limited for now) evidence derived from other organs that indicate that neutrophils could play positive roles, possibly in later stages of the pathology (Easton, 2013).
Neutrophils could be important in scavenging lesion-derived cellular debris; the production of MMP9 in later phases of the disease could participate in angiogenesis through vascular endothelial growth factor (VEGF) activation, and a systemic role in protection from post-ischaemic infections could be postulated (Jickling et al., 2015).
Recently it has been shown that the absence of Toll-like receptor 4 (TLR4) leads to increased numbers of infiltrating neutrophils, but it is associated with a beneficial effect since it drives polarization of these cells towards N2 phenotype, which is cytoprotective in stroke (García-Culebras et al., 2019).

Interfering with the infiltration of neutrophils is therefore a delicate matter, since their effect on the stroke outcome may be specific to different stages of the disease.

1.3.3. MACROPHAGES

Macrophages too can be considered early responders and contributors to the post-stroke inflammatory environment. Possibly due to their localisation in the immediate vicinity of the affected parenchyma, $CD163^+$ perivascular and meningeal macrophages have been shown to respond relatively quickly (16h) to ischaemia by promoting leukocyte chemotaxis, blood-brain barrier disruption and ultimately contributing to neurological impairment after stroke (Pedragosa et al., 2018).
Signs of peripheral infiltration have also been observed, in the form of peripheral monocytes that differentiate into macrophages upon entry into the brain parenchyma.
Monocyte infiltration has been detected by 24h after stroke onset, peaking at 3-4 days (Gelderblom et al., 2009). Of these, the first subtype to get access to the ischaemic brain are $CCR2^+Ly6C^{hi}$ pro-inflammatory monocytes that differentiate into Ly6Clo macrophages (Gliem et al., 2012; Miró-Mur et al., 2015).
The hypoxic milieu of the ischaemic brain and the presence of DAMPs induce a pro-inflammatory M1-like phenotype, mainly mediated by the activation of hypoxia inducible factor 1 (HIF-1) pathways (Cramer et al., 2003). In the acute phase of stroke, macrophages end up contributing to the ischaemic cascade by production of TNFα and IL-1β, while IL-23 and IL-12 production could contribute to the sub-acute involvement of inflammatory T cells (Nakamura and Shichita, 2019).
Since the modulation of the activation phenotype of infiltrating monocytes depends mainly on environmental stimuli, the progressive evolution of the infarct towards a resolution can also skew the differentiation of monocyte-derived macrophages towards an M2-like phenotype (Miró-Mur et al., 2015). This shift can contribute to the phagocytosis of dead cells and to the production of anti-inflammatory cytokines such as TGF-β and IL-10, that can indirectly contribute to a variety of resolutive mechanisms and ultimately to functional recovery (Pedragosa et al., 2020).

1.3.4. LYMPHOCYTES

Lymphocytes are increasingly recognised to be playing a role in influencing stroke outcome in the early phase (Chamorro et al., 2012), even though they infiltrate the ischaemic brain in lower numbers compared with innate cells (Gelderblom et al., 2009). The general view is of an early antigen-independent involvement of various

subsets of T cells, each with potentially detrimental or protective consequences (Gill and Veltkamp, 2016).
As happens for the innate cells, the infiltration of T cells to the brain after ischaemia is facilitated by the expression of inflammatory signals and adhesion molecules on blood vessels, as previously mentioned (chapter 1.2.1). In particular, post-capillary venules and the choroid plexus appear to be the main gateways for lymphocyte action and infiltration to the brain parenchyma (Schwartz and Baruch, 2014).
In human post-mortem samples, T cells are mainly found at the border of the infarct within days after ischaemia, while the infiltration and localisation of lymphocytes in animals appears to be model-dependent (Zhou et al., 2013).
There have been reports of very early infiltration, within 3 hours after onset, while the majority of studies detect lymphocytes starting at 24h after stroke and increasing 3 to 4 days after ischaemia (Gill and Veltkamp, 2016). All while a post-stroke lymphopenia occurs in the systemic circulation (Meisel et al., 2005).
The early effect of T cell presence in the brain vasculature appears to be independent of antigen presentation. The interaction of T cells with platelets and other leukocytes may be provoking thromboinflammatory intravascular reactions that negatively contribute to the outcome of ischaemia (De Meyer et al., 2016; Kleinschnitz et al., 2010).
Among the detrimental mechanisms mediated by classical T cells, the production of perforin by $CD8^+$ cells (Mracsko et al., 2014) and the secretion of IL-21 by $CD4^+$ T cells (Clarkson et al., 2014) are the most recognised. Overall, transgenic- and depletion-mediated deficiency of lymphocytes leads to smaller infarcts and better functional outcome in acute stroke models, but the dynamics of the deleterious role of classical T cells are still to be fully unravelled.
Forkhead box P3 positive ($FoxP3^+$) regulatory T cells (Treg) exert immunomodulatory functions that appear to be beneficial in stroke through IL-10-mediated actions (Liesz et al., 2009). These cells accumulate and proliferate in the ischaemic hemisphere up to 30 days after stroke (Stubbe et al., 2013) and complex interactions with other leukocytes can mediate their protective effect (Gill and Veltkamp, 2016). Nevertheless, also Tregs have been implicated in acute thromboinflammation (Kleinschnitz et al., 2013).
For the scope of this dissertation, particularly interesting is the role of gamma delta T cells (γδ T cells). These cells do not necessarily need clonal expansion to be activated, making them rapid responders in the immune response to both pathogens and tissue dysregulation, i.e. DAMPs release (Chien et al., 2014). One of the main pathways allowing antigen-independent activation is their constitutive expression of IL-23 receptor (Sutton et al., 2009). The main effector cytokines released by these cells (although in different proportions depending on their ontogeny) are interferon

gamma (IFNγ), TNFα and, most importantly in stroke, interleukin 17 (IL-17). Interestingly, a sustained IL-17 response appears to be dependent on both inflammatory cytokines signalling and TCR engagement, acting as a sort of self-regulator to obtain full response only in inflammatory conditions (Chien et al., 2014). In this dissertation we will mainly focus on natural γδ T cells, which develop in the thymus and colonize adult peripheral tissues, especially mucosal barriers (Chien et al., 2014). Various studies demonstrated the involvement of γδ T cells and IL-17 early after ischaemia, mediating subsequent neutrophil infiltration (Gelderblom et al., 2012b) and induction of MMP3 and MMP9 (Shichita et al., 2009). A more recent study demonstrated that the infiltration of these cells in the ischaemic brain depends on CCR6 functionality (Arunachalam et al., 2017). It has also been observed that these cells could be recruited all the way from the intestinal tract (Benakis et al., 2016). Interestingly, infiltration of these cells has also been demonstrated in ischaemic human brain tissue (Gelderblom et al., 2012b).

Overall, the early phase of acute stroke response appears to be driven by innate-like pro-inflammatory mechanisms, whereas the antigen-dependent adaptive reactions of lymphocytes may be more involved in a delayed phase (Gill and Veltkamp, 2016) **(Figure 11)**. More about their role in stroke will be explored in relation to their interaction with dendritic cells.

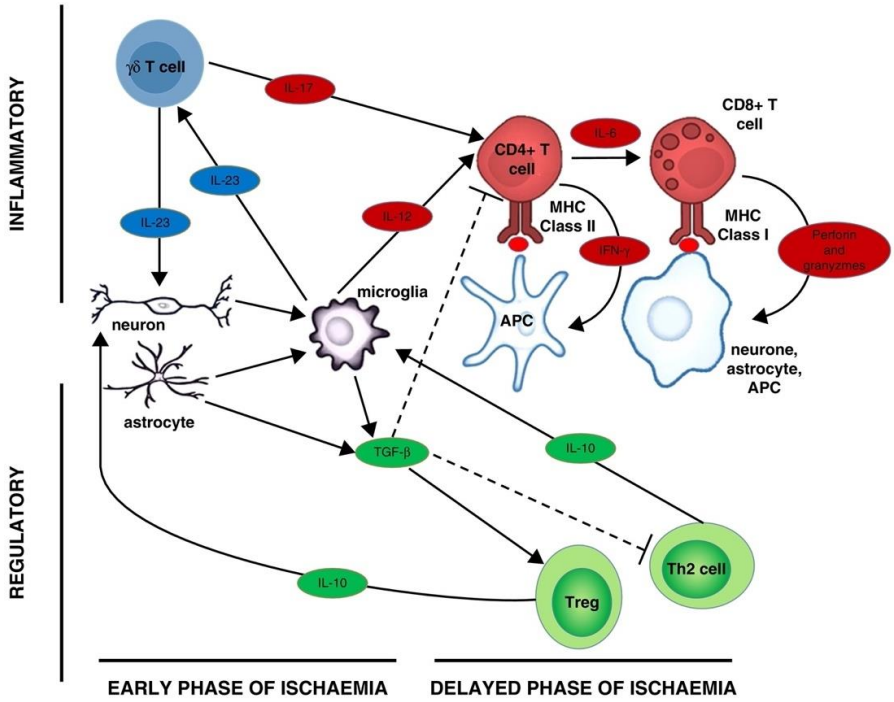

Figure 11. Involvement of T cells in cerebral ischaemia.
The first response by T cells is possibly driven by antigen-independent mechanisms (in blue) with a detrimental effect, while the delayed phase is more heterogeneous in outcome, with both inflammatory (red) and regulatory (green) cells involved. Source: (Gill and Veltkamp, 2016).

The involvement of all these immune cell types in the reaction to ischaemic stroke has led to various clinical trials aimed at reducing their nefarious roles (Gill and Veltkamp, 2016; Malone et al., 2019). Currently, the main focus of immunomodulation strategies is to limit the noxious acute phase of neuroinflammation, but we cannot forget that immune reactions continue for long after reperfusion and are also in part responsible for the resolution of the lesion and its possible repair, making the timing of the intervention a crucial aspect (Iadecola and Anrather, 2011).
Apart from the incomplete understanding of the immune reaction to stroke, the lack of significant breakthroughs in immunomodulatory approaches is possibly also due to the mismatch between the time-dependent and cell-dependent heterogeneity of the response and the limited specificity of the current pharmacological armamentarium available, which will possibly change soon with the most recent molecular approaches. Furthermore, it is important to keep in mind that the modulation of immunity towards a reduced reaction could act in synergy with the already ongoing immunosuppression, potentially aggravating the susceptibility to infections (Meisel et al., 2005).

1.4. STROKE-INDUCED IMMUNOSUPPRESSION

Upon stroke, both patients and experimental animals undergo a so-called stroke-induced immunosuppression (Meisel et al., 2005; Prass et al., 2003). It is thought to be due to an overactivation of the sympathetic nervous system and of the hypothalamic-pituitary-adrenal axis, which respectively produce catecholamines and glucocorticoids, both known anti-inflammatory mediators. Teleologically this response is supposed to be a defence mechanism that limits the inflammatory response in the brain, but since it is a systemic reaction, it can end up leaving the organism exposed to infections (Chamorro et al., 2007).
Apart from limiting the aggressiveness of the inflammatory response, it is possible that this reaction is a protection mechanism against autoimmune reactions directed at brain antigens, which are potentially liberated by the rupture of the BBB and dysregulation of the immune environment, and can reach the draining secondary lymphoid tissues (Planas et al., 2012).
However, the possibility of developing an infection makes this immunosuppression a double-edged sword, since it could lead to systemic inflammation and possibly make patients more prone to mounting effective immune reactions to brain antigens (Becker, 2012), in turn leading to worse neurological outcome (Becker et al., 2011).

Various cell types have been shown to be affected by stroke-induced immunosuppression. Neutrophils and monocytes from stroke patients displayed reduced capacity of oxidative respiratory burst in response to increased catecholaminergic signal (Ruhnau et al., 2014).
Monocytes have also been shown to decrease their expression of very late antigen-4 (VLA-4), which is responsible for their migration from the spleen (Offner et al., 2006), while increasing their expression of IL-10 (Urra et al., 2009).
The adaptive immune arm is also impaired, in part due to the apoptotic death of splenocytes, which leaves splenic T cells with a microenvironment poor in mitogenic factors (Prass et al., 2003). Apart from an impaired proliferation, T cells tend to be skewed towards a Th2 phenotype, possibly also due to the IL-10 production by monocytes (Chamorro et al., 2006).
The activation and stimulation of T cells can be further impaired by the altered signals that they receive from antigen-presenting cells like macrophages and dendritic cells, which have been shown to express reduced levels of MHCII molecules (Vogelgesang et al., 2008) and to be less effective at co-stimulation (Hug et al., 2011).
All in all, it is evident how the equilibrium of these reactions is very unstable between obvious benefits and potential risks, making therapeutic immunomodulatory interventions extremely difficult and thereby still largely unsuccessful.

2. DENDRITIC CELLS

Dendritic cells are antigen-presenting cells (APC) that act as fundamental regulators of both innate and adaptive immune responses. Their main role is to detect threats (both external and internal) to the homeostasis of the tissues and to mount a finely arranged response aimed at eliminating them, by combining and regulating the action of various effector mechanisms (Durai and Murphy, 2016).
Their involvement in stroke pathophysiology is being unravelled, thus an in-depth review of the current knowledge available is due.

2.1. ONTOGENY

In strict ontogenic and developmental terms, DCs include two main populations: conventional DCs (cDCs) and plasmacytoid DCs (pDCs).
Hematopoietic stem cells (HSCs) from the bone marrow continuously replenish the numbers of dendritic cells, since they have a short life and limited self-renewal capabilities (Ginhoux and Guilliams, 2016).
Common DC progenitors (CDPs) give rise to pDCs, which leave the bone marrow as terminally differentiated cells (Reizis, 2010), whereas cDCs develop from an intermediate pre-DC population, which migrates to the tissues before differentiating (Liu et al., 2009).
In mouse lymphoid tissues, pre-DCs differentiate into $CD8\alpha^{+}CD11b^{-}$ and $CD11b^{+}$ cDCs, whereas in non-lymphoid tissues they develop into $CD103^{+}CD11b^{-}$ and $CD11b^{+}$ cDCs. The ontogeny of these two subpopulations of cDCs in lymphoid and non-lymphoid tissues largely corresponds to a more recent nomenclature (which will be followed throughout this dissertation) that groups them as cDC1 and cDC2 respectively (Guilliams et al., 2014).
Interestingly, pre-DC- or cDC-committed CDPs can be identified by the expression of Dendritic Cell NK Lectin Group Receptor 1 (DNGR-1) (Schraml et al., 2013) and CD115, whereas pDCs appear to derive predominantly from $CD115^{-}$ CDPs (Onai et al., 2013). Lineage diversion at the level of CDP is mainly driven by the expression of different transcription factors.
pDCs complete their development in the bone marrow in E2-2-dependent manner (Merad et al., 2013). The expression of the transcription factor ZBTB46 is a marker of cDCs and their committed progenitors, even though it is dispensable for their development (Satpathy et al., 2012).
The development of cDC1 from their committed pre-DC progenitor depends on the transcription factors Irf8, Id2, Nfil-3 and Baft3, whereas cDC2 differentiation is controlled by Irf4, PU.1 and RelB (Pakalniškytė and Schraml, 2017).

Among these transcription factors, Irf8 and Irf4 are the ones that best allow the distinction of cDC1 and cDC2 subtypes across various tissues (Guilliams et al., 2016).

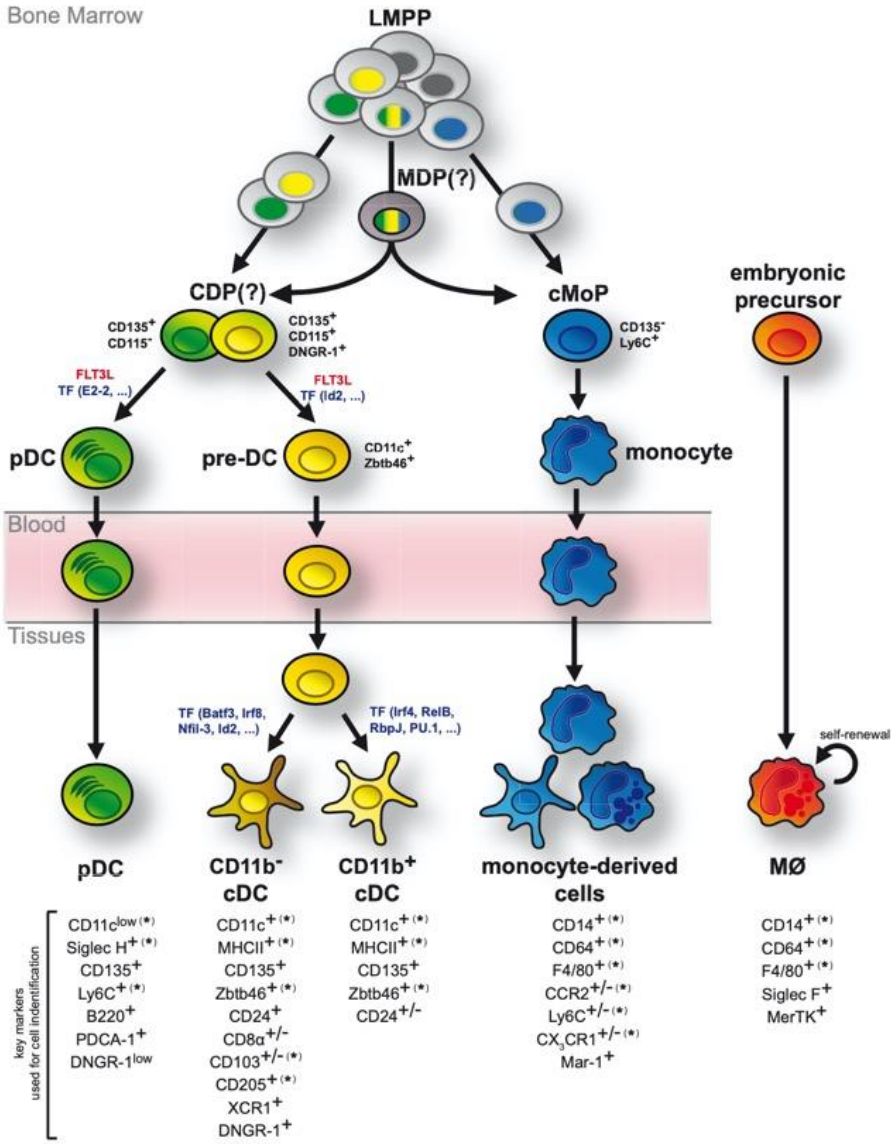

Figure 12. DC ontogeny, development and surface markers expression.
Lymphoid-primed multipotent progenitors (LMPP) exhibit partial commitment to the monocyte or DC lineage, giving rise to common DC progenitors (CDP) or common monocyte progenitors (cMoP), possibly through an intermediate monocyte and DC progenitor (MDP). Terminally differentiated DC subpopulations can be distinguished by the expression of a number of key markers, as indicated. Source: (Poltorak and Schraml, 2015).

Notably, some of the transcription factors that regulate DCs in mice appear to be involved also in the development of human DCs, and the separation of DC subpopulations appear to be conserved across species (Poltorak and Schraml, 2015).

Despite the relative consensus on this developmental relationships between subpopulations, the upstream regulation of the transcription factors responsible for the commitment to each terminal lineage and the exact step of development in which this commitment is established are still to be fully defined (Pakalniškytė and Schraml, 2017) **(Figure 12)**.

Apart from transcription factors, also cytokines can modulate DC development and homeostasis. All DC subsets depend on fms-like tyrosine kinase-3 ligand (Flt3L) for their development, since all the intermediate progenitors are Flt3$^+$ (Schmid et al., 2010).
Flt3L administration or genetic overexpression increase the number of both pDCs and cDCs (Maraskovsky et al., 1996), whereas gene deletion of Flt3L or Flt3 severely reduces it (Waskow et al., 2008).
The main role of Flt3L seems to be the peripheral expansion of DCs progenitors, but stimulating Flt3 downstream signalling in Flt3$^-$ progenitors can also per se differentiate them toward pDCs and cDCs (Onai et al., 2006).
Notably, the subpopulation most affected by changes in FLT3L levels seems to be the cDC1, both in homeostatic and pathological conditions (Guermonprez et al., 2013; Scott et al., 2014).
Other cytokines also play roles in the development of DCs. Colony stimulating factor CSF2 (GM-CSF) has shown involvement in the survival of non-lymphoid tissue cDC1 and cDC2 (Greter et al., 2012). Also CSF1 (M-CSF) can sustain DC development by stimulating the CD115 receptor present on CDPs and pre-DCs (Fancke et al., 2008), but M-CSF deficiency only mildly impacts these populations (MacDonald et al., 2005).

2.2. SURFACE MARKERS

Despite the recent advancements in DC development and characterisation, these cells are still mainly defined by their surface marker expression and by their functions in *in vivo* studies.
The historical broad definition of DCs relies on a variable degree of expression of the integrin CD11c and of MHC components, but these characteristics are often not precise or stable enough to distinguish DCs from other myeloid cells, especially in non-lymphoid tissues, and even less in inflammatory conditions (Guilliams and van de Laar, 2015).
Advancements in multi-colour flow cytometry allowed the integration of ontogenic and functional information with surface characterisation of DCs, adding weapons to the surface marker arsenal needed to define DCs and separate DC subpopulations.

The main advancement was arguably the distinction of cDC1 from cDC2 by the mutually exclusive expression of XCR1 and CD172, first demonstrated in the intestine (Becker et al., 2014) and later expanded to other numerous mouse organs (Gurka et al., 2015).
This phenotypic separation correlated with the different ontogeny and transcription factor dependency of the classical DC subsets described earlier (Guilliams and van de Laar, 2015). These markers are not exclusive to DCs, so it is important to stress the necessity of defining the broad population of DCs with more canonical markers prior to the separation of subpopulations.
pDCs can be separated mainly by their expression of B220, Siglec-H or Bst2, but they have to be first identified by their low expression of CD11c and MHCII (Reizis et al., 2011).
Another important and necessary separation is the one that sets monocyte-derived DCs (moDC) apart from conventional DCs. moDCs are a very heterogeneous population derived from common monocyte progenitors (CMoP), and a great deal of the available knowledge about them comes from *in vitro* studies that are known to have influenced some results (Mildner and Jung, 2014). *In vivo* these cells appear to be present only in inflamed tissues (Segura and Amigorena, 2013).
The main surface marker separating moDCs from cDCs in mice is considered to be CD64 (Langlet et al., 2012; Tamoutounour et al., 2012), especially in combination with FcεRI (Plantinga et al., 2013). Nevertheless, a more recent study identified a $CD64^+$ Flt3L-dependent population classifiable as cDC in the inflamed lymph nodes (Min et al., 2018).
The expression of Ly6C is also useful to discriminate between DC subpopulations, since only moDCs and pDCs express it (Amon et al., 2019).

2.3. FUNCTIONS

2.3.1. RESPONSE TO PATHOGENS

Functionally, DCs are considered the pivotal population responsible for T cell stimulation and initiation of adaptive immune responses (Banchereau and Steinman, 1998).
By presenting intracellular antigens or cross-presenting exogenous antigens on MHCI, cDC1 are able to prime CD8 cells, while cDC2 are responsible for the activation of CD4 cells through MHCII-mediated antigen presentation (Dudziak et al., 2007).

The expression of MHCII on cDC2 has also been associated to the reception of a licensing signal by activated CD4 T cells, that in turn enhances the priming of antigen-specific CD8 cells (Eickhoff et al., 2015).
Upon contact with foreign antigens, DCs undergo a maturation process that allows them to be most efficient in stimulating the T cell response, mainly producing co-stimulatory molecules (CD80, CD86, CD49 and others), upregulating migratory chemokine receptor such as CCR7 and secreting stimulatory cytokines such as IL-12, TNFα and others.
The T cell stimulation then happens in the draining lymph nodes, from where the primed T cells emerge to migrate to the tissue of destiny as effector cells (Amon et al., 2019).
Besides T cell priming, dendritic cell subsets are able to induce different types of immune responses, mainly through cytokine release (Durai and Murphy, 2016; Hilligan and Ronchese, 2020) **(Figure 13)**.

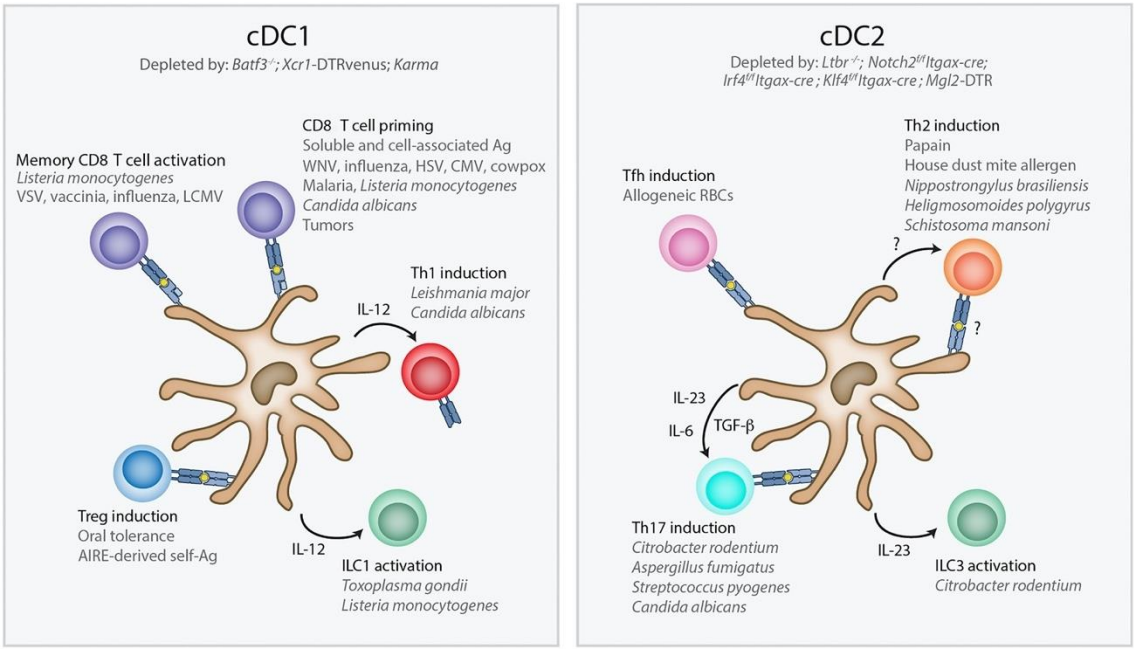

Figure 13. Functions of dendritic cells in immune response.
Each subset is responsible for a different class of Th response. cDC1 are mostly responsible for Th1 and Treg induction. cDC2 are critical for Th17 induction and possibly Th2. Source: (Durai and Murphy, 2016).

cDC1 are important in the induction of type I immune responses mounted against intracellular pathogens: TLR3- and TLR9-enhanced IL-12 release by cDC1 is crucial for the Th1 differentiation of T cells and for the activation of type 1 innate lymphocytes and NK cells that produce IFNγ, which in turn stimulates the defensive response by macrophages and feedbacks on DCs to further produce additional IL-

12. These macrophages and cytotoxic CD8 T cells primed by cDC1 are the main effectors responsible for the clearance of intracellular pathogens.
Monocyte-derived dendritic cells have also been associated to Th1 responses to infections through their production of IL-12 (Hilligan and Ronchese, 2020) and more recently a TNFR2$^-$ subset of cDC2 has been implicated too (Mansouri et al., 2019).
The role of DCs in type II immune responses against parasites or allergens is less defined. The Th2 polarization of T cells is a crucial factor, and several genetic cell ablation studies demonstrated it is influenced by cDC2. The mechanism by which cDC2 may polarize Th2 cells is not clear, but it may involve type 2 innate lymphocytes (Halim et al., 2016). On the contrary, cDC1 hinder Th2 polarization through their constitutive production of IL-12 (Hilligan and Ronchese, 2020).
Type III immune responses are mainly Th17-mediated and are directed against extracellular bacteria and fungi. Several DC-produced cytokines are involved in these processes: among them IL-23, IL-6 and TGF-β. The contribution of type 3 innate lymphocytes is also mediated by IL-23. Several cell ablation approaches allowed to pinpoint cDC2 as the main regulators of Th17 differentiation, with a possible cooperation by moDCs (Hilligan and Ronchese, 2020). The pathways involved in these mechanism are varied, but particularly TLR2 engagement appears to be a discriminating signal for DC influence towards Th17 polarization since it skews DC cytokine production from IL-12 to IL-23, at least in humans (Re and Strominger, 2001).

2.3.2. TOLERANCE TO SELF-ANTIGENS

Apart from the response to pathogens, DCs are fundamental in maintaining tolerance to self-antigens. Early DC-depleting mouse models were used to demonstrate this concept: eliminating DCs was sufficient to induce signs of autoimmune pathology such as splenomegaly and lymphadenopathy (Audiger et al., 2017).
On the contrary, the effect of increasing the number of DCs is not straightforward and depends on the method used to achieve it. FLT3L injection induces an increase in DCs and prevents autoimmune diabetes development in NOD mice, whereas inhibition of DC apoptosis leads to accumulation of immune cells in non-lymphoid organs and ultimately inflammation (Audiger et al., 2017).
Various subsets of DCs contribute substantially to the process of negative selection of thymocytes by expressing self-antigens and cross-presenting blood antigens on their MHC molecules, thus participating in central tolerance establishment (Hogquist et al., 2005).

In addition to this, thymic DCs are also responsible for the induction of natural antigen-specific Tregs, both by driving their differentiation and by sustaining their survival via CD70 expression (Audiger et al., 2017) **(Figure 14)**.

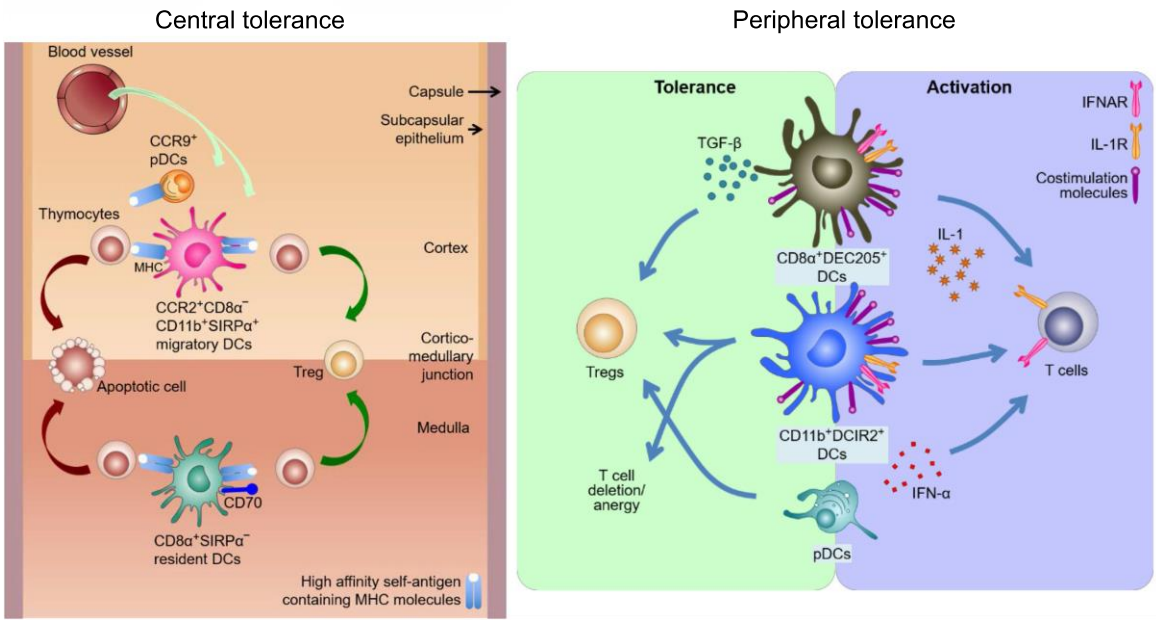

Figure 14. DC-mediated central and peripheral tolerance.
In the thymus and in the periphery pDCs and cDCs induce tolerance by apoptosis/anergy of self-reactive T cells or by inducing Treg differentiation or activation. Source: (Audiger et al., 2017).

However, central tolerance is not enough to guarantee the prevention of autoimmune reactions in peripheral tissues. The T cell repertoire still contains self-reactive T cells even after negative selection in the thymus, and these cells can be activated by antigen cross-reactivity or by specific conditions that lower their affinity-dependent activation threshold (Iberg et al., 2017).

Peripherally induced T cell tolerance is therefore a crucial component of the immune homeostasis maintenance, and DCs are master regulators of this process. In steady-state condition, the absence of secondary costimulatory signals leads to anergic reactions or deletion of T cells. Where a steady-state condition is difficult to achieve due to constant antigen exposure, such as in the intestine, Tregs play a crucial role in the establishment of peripheral tolerance; here cDC1 and, to a lesser extent, a small cDC2 subset have been shown to drive regulatory T cell polarization, mainly through the expression of B- and T-lymphocyte attenuator (BTLA) and TGF-β (Esterházy et al., 2016).
This adds up to previous data showing that organ-specific tolerance and Treg induction is reduced in *Batf3*$^{-/-}$ mice, although no spontaneous autoimmune phenotype is evident. DC expression of molecules capable of interacting with CTLA-

4 and PD-1, and the production of retinoic acid have also been implicated in their capacity to induce Treg cell function.
Altogether, the role of cDC1 cells in Treg-mediated peripheral tolerance seems prominent, albeit not exclusive, but there is still no clear phenotypic or functional definition of a specifically tolerogenic subset of DCs, which could instead represent a temporary functional specialization (Ardouin et al., 2016; Iberg et al., 2017).

Apart from the more typically studied role of DCs as antigen-presenting cells, new ways in which these cells can modulate the immune response are being investigated. Among these, it is worth mentioning the sensing of cell death-derived molecules (i.e. DAMPs) via DNGR-1. DNGR-1 (encoded by the gene *Clec9a*) is a dendritic cell-specific receptor, and more specifically it is a marker of *Batf3*-dependent cDC1 cells (Poulin et al., 2012). It senses filamentous actin exposed upon necrotic cell death (Zhang et al., 2012). Besides its crucial role in cross-presenting antigens to $CD8^+$ cytotoxic T cells (Sancho et al., 2009), DNGR-1 has also been shown to regulate inflammation by limiting neutrophil infiltration in models of both sterile and infectious diseases. Its action was carried out independently of the adaptive immune compartment, and its role in disease tolerance has thus been proposed (del Fresno et al., 2018).
In a model of bacterial infection of the skin, cDC1 cells produced vascular endothelial growth factor α (VEGF-α), which is a recruiter of neutrophils in the skin (Janela et al., 2019), in apparent contrast with the previously reported results. In the gut, DCs participate in the maintenance of commensal microbiota homeostasis by sensing bacteria through Mincle and regulating the action of γδ T cells (Martínez-López et al., 2019).
This complexity of action underlines the necessity to better understand the relative contribution of DCs and their subsets in different organs and inflammatory settings.

2.4. BRAIN DCS

Compared with other organs, knowledge about brain DCs is largely underdeveloped, possibly due to the long-standing concept of the brain as an immunologically privileged site and to the low brain DC numbers under physiological conditions. But precisely because the brain presents such a strictly regulated immune environment, it is reasonable to think that DCs may play a crucial regulatory role in this organ.
In steady-state conditions dendritic cells have been observed mainly in the meninges, choroid plexus and circumventricular organs of both rodents and humans, seldomly appearing in the brain parenchyma (D'Agostino et al., 2012). With the implementation of transgenic animal models where the expression of

enhanced yellow fluorescent protein (eYFP) was driven by the integrin CD11c promoter (CD11c-eYFP mice), it was possible to more easily observe CD11c$^+$ cells (Lindquist et al., 2004); nevertheless, it is important to keep in mind that the expression of this marker is not exclusive to DCs, since CD11c can be expressed in a variable degree by other immune cells, among which most importantly monocytes and microglia (D'Agostino et al., 2012; Dando et al., 2016; Kamphuis et al., 2016).
Seminal results by the group of Steinman and Nussenzweig taking advantage of this transgenic animal identified CD11c-eYFP$^+$ cells within the developing or steady-state adult brain parenchyma (Bulloch et al., 2008). The eYFP-labelled cells colocalized with other immune surface markers such as CD11b, Iba-1 and F4/80, typically also expressed by macrophages and microglia. eYFP$^+$ cells were observed in regions of active synaptic plasticity or neurogenesis, such as the subventricular and subgranular zones, but also in border zones where the communication with the periphery is most direct due to the lack of a strict BBB, such as the circumventricular organs, the meninges and the choroid plexus (Bulloch et al., 2008). Interestingly, a later study observed CD11c-eYFP cells also in the juxtavascular parenchyma, with elongations integrated in the glia limitans, a position compatible with a patrolling function of the border environment (Prodinger et al., 2011). Another study detected DNGR-1$^+$ DCs in the meninges and choroid plexus of steady-state mice (Quintana et al., 2015).
Since it has been shown that only the eYFP$^+$ cells observed in the brain parenchyma colocalized with Iba-1, while the ones observed in the choroid plexus and meninges did not, it is possible that the former could be microglia-derived; this could be due to the capacity of microglia to express CD11c under specific conditions (Butovsky et al., 2007; Kamphuis et al., 2016; Prodinger et al., 2011), which very recently led some to propose it as a specific microglia subpopulation (Benmamar-Badel et al., 2020).
Apart from expressing CD11c, microglial cells can also acquire antigen-presenting characteristics upon specific stimuli such as IFN-γ intracerebral injection (Gottfried-blackmore et al., 2009) or *ex vivo* granulocyte-macrophage colony-stimulating factor (GM-CSF) exposure (Fischer and Reichmann, 2001), complicating the discrimination of these populations.
The eYFP$^+$ cells present in the meninges and choroid plexus of the steady-state brain were further characterised as bona fide dendritic cells by their dependence on Flt3L and their capacity to present antigen (Anandasabapathy et al., 2011).

Since the available knowledge on the involvement of DCs in stroke is limited, I will now introduce relevant evidence of their role in other neurological diseases, from which several concepts can be transposed to the cerebral ischaemia setting.

2.4.1. DCs IN NEUROINFLAMMATION

A neuroinflammatory context complicates even more the analysis of the role of brain dendritic cells, mainly because of changes in surface marker expression by microglia and macrophages. Inflammatory activation of these cells typically induces the expression of CD45, CD11c and MHCII, three of the main markers used to define DCs in the brain (Butovsky et al., 2007; Fischer and Reichmann, 2001; Gottfried-blackmore et al., 2009). Furthermore, monocyte-derived DCs appear in inflamed tissues, and the brain is no exception (Greter et al., 2005). The bulk of knowledge accumulated to date mainly relies on methods that did not always discriminate between cDCs, moDCs or DC-like resident and infiltrating cells; nevertheless, the body of evidence for the presence and role of antigen-presenting cells in various neurological diseases is quite large and has been extensively reviewed (Colton, 2013; D'Agostino et al., 2012; Ludewig et al., 2016) **(Figure 15)**.

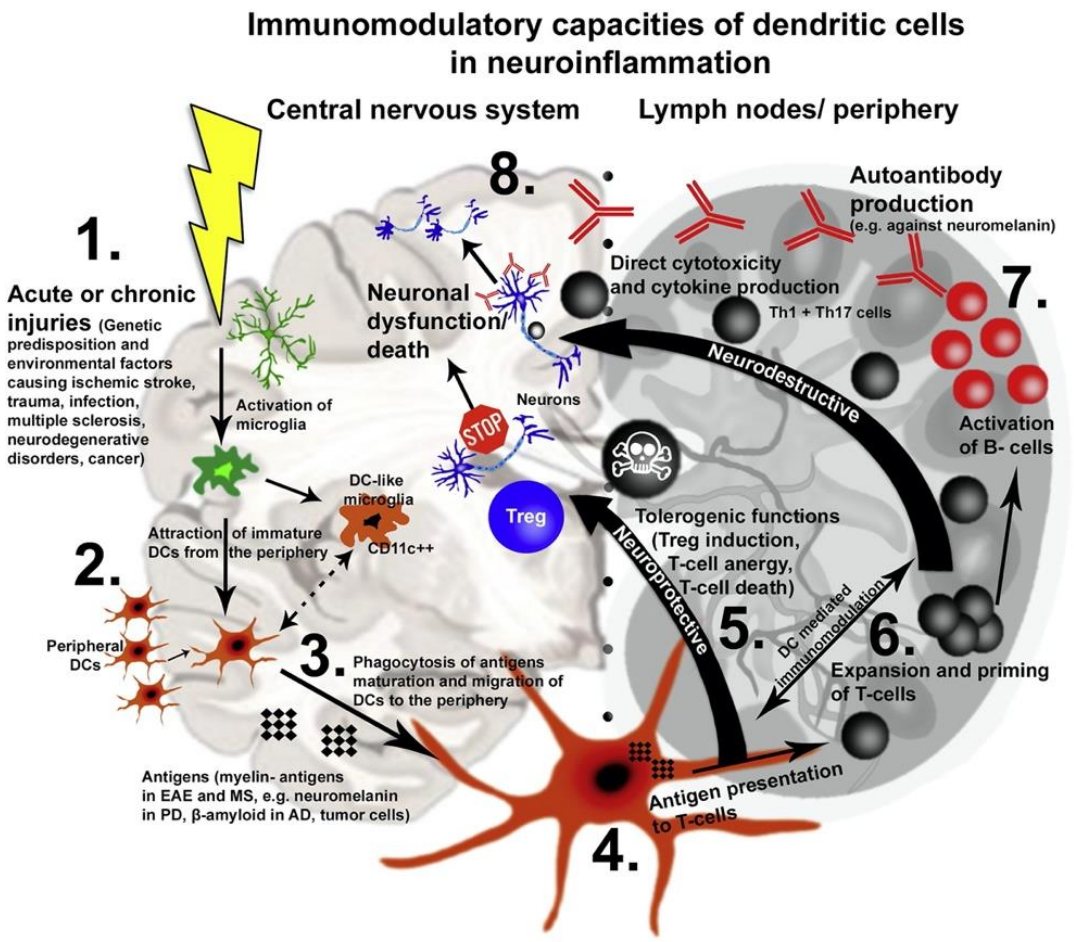

Figure 15. Dendritic cells in neuroinflammation.
This illustration resumes various points of actions of DCs in neuroinflammatory conditions. (1.) Patrolling or resident DCs react to local inflammatory cues. (2.) Peripheral DCs infiltrate the lesioned tissue and activate. (3.) DCs phagocytose antigens and migrate to secondary lymphoid tissues. (4.) DCs mature and present antigens in the draining secondary lymphoid organs. (5.) DCs can promote neuroprotective functions by inducing tolerance to self-antigens or (6.) could mediate the stimulation of Th1 and Th17 cells. (7.) The production of autoantibodies can also be mediated by indirect stimulation by DCs. This ultimately could lead to exacerbation of the tissue damage (8.). Source: (Ludewig et al., 2016).

Leaving infectious diseases aside, one of the most studied neurological inflammatory disease is multiple sclerosis, where the role of DCs is considered crucial because of its autoimmune origin.
DCs appear to be crucial in polarizing T cells in experimental autoimmune encephalomyelitis (EAE), the mouse model of multiple sclerosis, but since this model can be produced by different mechanisms, the role of other antigen-presenting cells different from DCs has also been observed (Ganguly et al., 2013). Importantly, DCs may intervene in two different steps of the pathology: during the priming of self-reactive T cells and during the restimulation phase, which is arguably the one most directly responsible for the encephalitogenic activity of T cells.

Nevertheless, ablation of DCs did not alter the priming of encephalitogenic T cells. In fact, mice constitutively lacking DCs showed a worse pathological outcome compared with control mice, highlighting how DC-mediated tolerance to self-antigens is crucial, and how other APCs may take up the immunogenic role of DCs in EAE (Isaksson et al., 2012; Yogev et al., 2012).
Microglia have been shown to play a pathogenic role in EAE, since microglia-deficient mice showed a delayed offset of the pathology and better clinical outcome (Greter et al., 2005).
Myelin antigens were observed already in steady-state microglia, with an increase upon EAE initiation, while infiltrating DCs only acquired antigens at symptom onset, hinting at the possibility that the two cell populations play different, complementary roles: while the migratory capacity of DCs may confer them an advantage in T cell priming capabilities, microglia may be more critical for the local modulation of the response (Sosa et al., 2013).

Another example of sterile brain pathology where DCs may play key roles is represented by brain tumours. The tumour microenvironment is a challenging setting for antigen presentation, since brain resident cells generally express low levels of MHC molecules and their malignant transformation further hinders their capacity (Ludewig et al., 2016). Nevertheless, a complex interplay of DCs, microglia, infiltrating macrophages and T cells is evident and currently under active investigation (Srivastava et al., 2019).
The main, most obvious role for dendritic cells in this category of brain disease would be the uptake and presentation of tumour antigens locally in the brain or in the draining cervical lymph node to stimulate T cell activation and tumour cell elimination. However, the particularly immunosuppressive milieu of gliomas complicates the classical development of the immune response. Vascular endothelial growth factor (VEGF), prostaglandin E_2 (PGE_2), interleukin 10 (IL-10) or CSF1 have all been shown to inhibit DC activation and skew their differentiation towards tolerising/suppressing phenotypes, which limit the recruitment of $CD8^+$ cytotoxic lymphocytes or stimulate the activation of Tregs, ultimately leading to the tumour immune evasion (Srivastava et al., 2019). As an example, the glioblastoma microenvironment is thought to stimulate the nuclear factor erythroid 2-related factor 2 (Nrf2) pathway in DCs, resulting in suppression of their maturation. The inhibition of this pathway was able to rescue CD80 and CD86 co-stimulatory molecule expression and to restore the production of cytokines (Wang et al., 2017). These immunoregulatory mechanisms by Nrf2 have also been proposed in stroke, together with other actions such as the control of oxidative stress (Liu et al., 2019).

Interestingly, microglia may also be involved in similar mechanisms of tumour evasion, mainly by inducing T cell anergy, due to their limited expression of co-stimulatory molecules (Matyszak et al., 1999).

Neurodegenerative disorders also often elicit immune responses in the CNS.
Again the first responder seems to be the resident microglia, which have been observed for example surrounding senile plaques in Alzheimer's disease, but also DCs undergo a rapid expansion (Ludewig et al., 2016).
Neurodegenerative diseases often present an increase in BBB permeability, leading to an easier egress of brain-sequestered antigens, which can be taken up by antigen-presenting cells that can then migrate to the lymph nodes to start an adaptive immune response; this can lead for example to inflammatory pathologies such as cerebral amyloid angiopathy or Aβ-related angiitis, which are triggered by the presence of Aβ antigens in brain endothelial cells.
Interestingly, *in vitro* analysis of DCs from patients with Alzheimer's disease showed that these cells have a reduced capacity of presenting antigen due to a reduction of MHC-related molecules, possibly providing a mechanism by which Aβ peptides can evade the immune response.
DCs may also be involved in Parkinson's disease (PD) pathogenicity, since a reduction of blood DCs has been observed in patients and has been interpreted as a consequence of recruitment to the brain (Ciaramella et al., 2013). Furthermore, the finding of autoantibodies against targets associated with PD pathology, such as neuromelanin or α-synuclein, suggests that autoimmune responses may be elicited by the presentation of these antigens in cervical lymph nodes (Ludewig et al., 2016).

One important issue in the reaction of DCs to neuroinflammation is their migration both to and from the brain, which is crucial for their action and is to date not completely understood.
DCs express a wide range of chemokine receptors, both of the C-C family (CCR1, 2, 3, 4, 5, 6, 7) and of the C-X-C family (CXCR2 and 4) (Alvarez et al., 2008).
This set of receptors makes DCs particularly sensitive to various inducible chemokines such as CCL2, 3, 4, 5, 7, 19, 20 and 21. Some of these are expressed at low level in homeostatic conditions and are responsible for the presence of few DCs with patrolling functions in peripheral organs (De Laere et al., 2018). CCRs from 1 to 6 are most important in the response to inflammation, which increases the levels of chemokines that are ligands for these receptors. Once they uptake antigens, DCs undergo a complex process of maturation that allows them to migrate to secondary lymphoid organs to mount an adaptive immune response. Different routes have been described for DC egress from the brain: (i) via the blood stream upon crossing

the perivascular space; (ii) along the rostral migratory stream via the cribriform plate, following olfactory nerves; (iii) via CNS drainage through the recently described dural lymphatic vessels. This migration is mostly driven by CXCR4 expression and strong upregulation of CCR7, which together make them move towards gradients of CXCL12, CCL19 and CCL21 chemokines, mostly expressed in lymphoid organs (Alvarez et al., 2008).

Most of the knowledge about migration to and from the brain in pathological situations is derived from the rodent multiple sclerosis model, EAE, but can be extrapolated more generally to inflammatory pathologies of the brain. Inflammation of the brain, as in other organs, induces the expression of DC-attracting chemokines such as CCL2, 3, 4, 5 and 19, which have been observed both in the parenchyma and cerebrospinal fluid (CSF) of patients and experimental models. Concomitantly, DCs upregulated some of the aforementioned receptors in this disease (De Laere et al., 2018).

The CCL2-CCR2 axis has been one of the most studied, with the crucial observations that CCR2-deficient mice are resistant to EAE and that CCL2-deficient mice develop a milder pathology. The source of CCL2 in the brain was identified in glial cells and susceptibility to developing EAE was ascribed to peripheral CCR2-expressing antigen-presenting cells (De Laere et al., 2018).

While the drivers of DC infiltration in the parenchyma upon inflammation have been mostly elucidated, the access of these cells to the CSF is mechanistically more dubious. It is known that even the steady-state CSF contains chemokines able to attract DCs and that these cells are present in low numbers, but the mechanisms of their migration are poorly understood (De Laere et al., 2018) **(Figure 16)**.

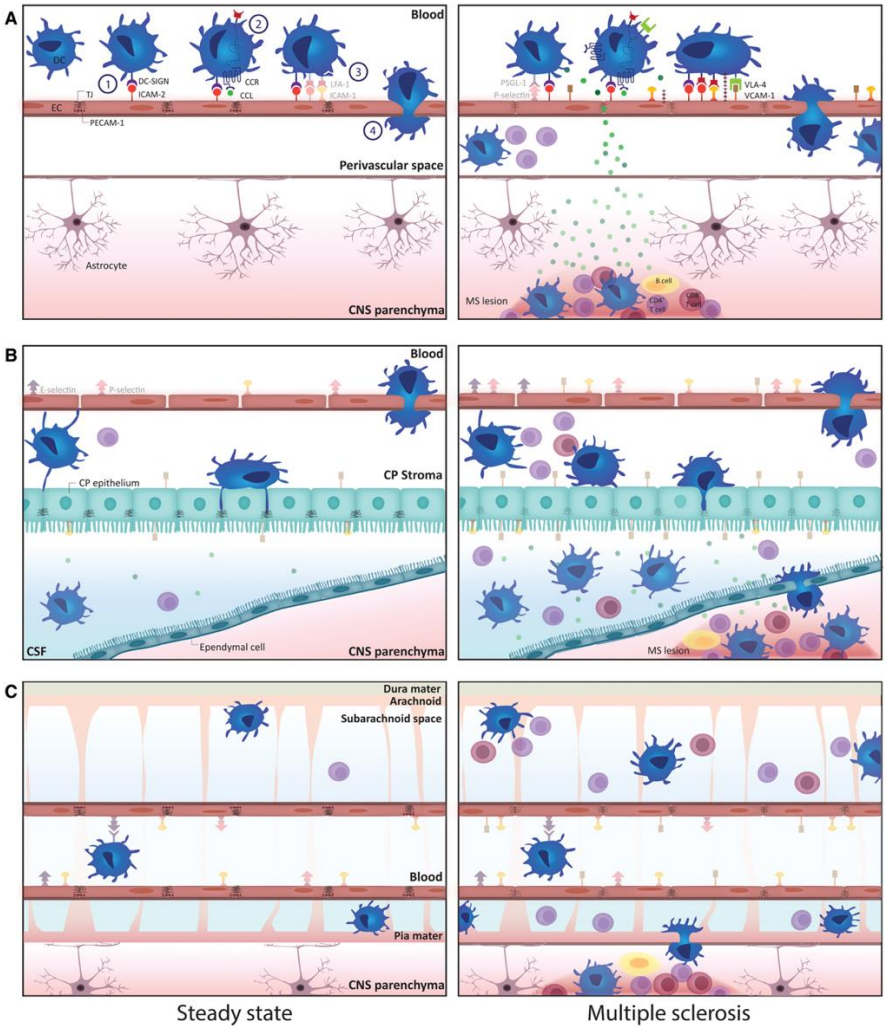

Figure 16. Migration of DCs into the brain.
Schematic overview of the infiltration of DCs via different compartments of the CNS. (A) In steady-state condition, DCs circulate in the blood stream and they only infiltrate the perivascular spaces. In case of inflammation, they are able to better adhere to the endothelium and cross the BBB, reaching the parenchyma. (B and C) DCs can be observed in the choroid plexus and meningeal spaces in steady-state condition. Upon inflammation their number increase and they can reach the brain parenchyma via these routes. Source: (De Laere et al., 2018).

2.4.2. DCs IN STROKE

In stroke, the pathological environment described in chapter 1.2.1 represents a prototypical stage for the involvement of antigen-presenting cells: infiltration of inflammatory cells, BBB breakdown, brain antigen egress, presence of danger signals, all could potentially be cogs that set the adaptive immune machinery in motion.

The presence of cells with features of DCs has been reported both in human patients and in rodent models of stroke. Kostulas et al. were among the first to detect in rats an increase of cells expressing the lymph node DC marker OX62 (CD103 in mice)

upon permanent middle cerebral artery occlusion (pMCAo). They also observed that these cells were marked by OX6, which recognises MHCII, and OX42, which corresponds to CD11b in mice. Upon demonstrating a correlation between the number of $OX62^+OX42^+$ cells and the infarct size, they underlined the role DCs may play in the pathology, but did not investigate the origin of these cells, leaving open the possibility that they may derive from microglia differentiation (Kostulas et al., 2002).

In the same year Reichmann et al. demonstrated the presence of $CD11c^+$ cells in the ischaemic brain of mice, observing an increase already 24 hours after ischaemia.

Again, the concomitant expression of other myeloid markers impeded a clear classification, but the elevated level of CD45 expression of the cells closest to the infarct area led to conclude that at least some of them were blood-borne (Reichmann et al., 2002).

Some years later, Gelderblom et al. showed, in a tMCAO model of ischaemia in mice, the temporal and spatial dynamics of infiltration of various immune cells in the brain. Among other cells, they observed a sharp increase of $CD11c^+MHCII^+$ cells, starting at day 1 after stroke and peaking around day 3 (Gelderblom et al., 2009).

Felger et al. used CD11c-eYFP transgenic mice to demonstrate the accumulation of $eYFP^+$ cells in the ischaemic tissue of mice subjected to tMCAO. Using radiation bone marrow chimeras, they showed that infiltrating $eYFP^+$ cells localised in the core of the infarction, while radiation-resistant cells localised in the penumbra, hinting at the heterogeneity of the CD11c-expressing population in the brain (Felger et al., 2010) **(Figure 17)**.

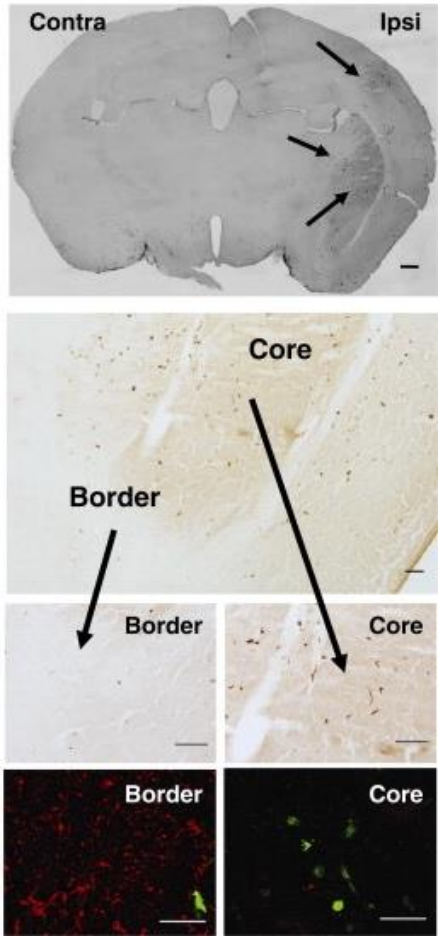

Figure 17. Presence of peripheral eYFP+ cells in the ischaemic brain.
One of the first demonstration of the infiltration of peripheral CD11c-eYFP cells in the infarcted brain of mice. Upper panel: 3,3′-Diaminobenzidine (DAB) immunohistochemistry for eYFP+ cells, which concentrate near the core of the infarct at 72h. Middle panel: higher magnification of DAB immunohistochemistry. Small lower panels: immunofluorescence for glial fibrillary acidic protein (GFAP, red) and eYFP. Scale from top to bottom: 400µm, 100µm, 100µm and 50µm. Source: (Felger et al., 2010).

Several other studies in mice and rats observed increased numbers of DCs in various models of cerebral ischaemia and the same observations were made in human patients, but none of these studies investigated in detail the origin and identity of DCs (Ludewig et al., 2016).

What also remains elusive is the role DCs play in the immune response to the ischaemic insult in the brain, even though experimental manipulation of their number or activity has provided some clues. For example, treatment with the flavonoid fisetin reduced the number of CD11c+ cells and hampered the activation of microglia 3h after tMCAO, leading to smaller ischaemic lesions compared with control-treated mice (Gelderblom et al., 2012a).

In another study, treatment with levodopa, whose D1 receptor is expressed by a large fraction of MHCII$^+$ cells, led to a reduction of the expression of MHCII in the ischaemic core and to a consequent amelioration of white matter tract degeneration (Kuric and Ruscher, 2014).
Also, treatment with granulocyte colony stimulating factor (G-CSF) has been shown to suppress dendritic cell functions and to ameliorate cerebral inflammation, infarct size and neurological outcome in rats (Dietel et al., 2012). However, there are also reports of possible beneficial effects of DC activity in stroke: peripheral CD11c$^+$ cells have been shown to upregulate the expression of indoleamine 2,3-dioxygenase (IDO) upon experimental ischaemia; IDO is known to favour functional development of Treg, which could ultimately participate in immunoregulation in the ischaemic brain (Wang et al., 2015).
These results indicate that DCs, or more generally antigen-presenting cells, certainly play a role in stroke, but their influence on the outcome of the pathology is still unclear.

2.4.3. ANTIGEN PRESENTATION AND SELF-REACTIVITY

In stroke, APCs may stimulate a T cell response against antigens that escaped the immunologically privileged environment of the brain upon BBB breakdown or upon uptake and migration to the cervical lymph nodes by the very same APC. This is suggested by cumulative evidence of the presence of brain antigens and antigen-presenting cells in the lymphoid tissues draining the CNS of stroke patients and experimental models (Javidi and Magnus, 2019; Miró-Mur et al., 2016; Urra et al., 2014).
Interestingly, brain antigens were detected in APCs in the draining lymphoid tissue of stroke patients; antigen-specific consequences can either lead to better or worse outcome depending on whether the antigen is neural- or myelin-derived (Planas et al., 2012). It was observed that myelin-specific T cells infiltrate the brain after stroke, by injecting T cells stimulated *in vitro* with myelin oligodendrocyte glycoprotein (MOG) peptides into severely immunodeficient mice (Ren et al., 2012). Pivotal experimental studies demonstrated that induction of mucosal tolerance to myelin basic protein (MBP) prior to stroke reduced the size of the brain lesion (Becker et al., 1997).
The presence of a self-reactive response by T cells after stroke was also observed by administering a recombinant T cell receptor ligand that specifically targeted myelin-specific T cells and inactivated them. This in turn reduced the overall inflammatory reaction of the infarcted brain and the infiltration of immune cells (Subramanian et al., 2009). Also the timing of T cell infiltration in the brain makes it plausible to

think that some kind of self-reactivity is being developed, since it has been observed starting at 4 days after stroke (Ortega et al., 2015).
More recently it was observed that T cells of 2D2 transgenic mice, where 80% of T cells are specific for MOG, locally react after stroke to MOG peptides presented by microglia, exacerbating the Th1 and Th17 response in the brain and ultimately leading to worse outcome (Jin et al., 2018). However, a very recent study by our group in stroke patients did not detect T cell responses to neural-antigen stimulation 90 days post-stroke (Miró-Mur et al., 2020). The study showed acute T cell priming in the periphery and T cell trafficking from the CSF to the ischaemic brain tissue, but it suggested the existence of active mechanisms preventing self-reactivity (Miró-Mur et al., 2020).
Indeed, despite these several pieces of evidence, an overt autoimmune response has never been observed, possibly because of the systemic immunosuppression that develops soon after stroke, introduced in chapter 1.4 (Chamorro et al., 2007; Meisel et al., 2005).
Interestingly, it has been shown that preventing stroke-induced immunosuppression increased the response of CNS-specific T cells in the brain of 2D2 mice, but this did not lead to a worse functional outcome (Römer et al., 2015).
However, if this brain-protecting immunosuppression mechanism leads to an infection, the systemic immune environment would be subject to another 180° turn towards activation and response.
Brain-derived antigen presentation can then be happening in two very different systemic conditions that may be influencing its ability to stimulate an effective adaptive response: on the one hand we can have an acute systemic immunosuppression that reduces the ability to present antigen in a co-stimulatory pro-inflammatory condition, and on the other hand we can have a secondary inflammatory situation due to infection that can favour a more effective stimulation and priming of brain-reactive T cells.

Autoantibodies against brain antigens, mainly IgA and IgE, have been found in the cerebrospinal fluid and serum of stroke patients, but their clinical relevance is still unclear (Miró-Mur et al., 2016). Zerche et al. reported pre-existing autoantibodies against the NMDA receptor 1 (IgM, IgA and IgG) that were beneficial or detrimental depending on the status of the BBB (Zerche et al., 2015). However, other studies that also detected some pre-existing autoantibodies in the blood of stroke patients did not find relationships with the clinical outcome, and did not find induction of new autoantibodies in stroke patients (Royl et al., 2019), leaving the role of autoantibodies in stroke to a more relegated stage.

Hypothesis and Objectives

HYPOTHESIS

After ischaemia, different dendritic cell subpopulations infiltrate the brain and carry out specific functions that need to be unravelled to better understand the neuroinflammatory processes that take place in the ischaemic tissue. Furthermore, dendritic cells infiltrating the murine brain after ischaemic stroke display distinctive characteristics that allow to discriminate them from microglia.

OBJECTIVES

The main objective of this work is to differentiate the contribution of specific infiltrating dendritic cell subpopulations to the pathophysiology of brain ischaemia in mice. Furthermore, we aim to identify both phenotypic and functional peculiarities of DCs to distinguish them from resident microglia.

To this end, we divided the project into several intermediate objectives:

1. To characterise the subpopulations of DCs infiltrating the ischaemic brain in the acute phase.
2. To investigate the role and mechanism of action of DC subpopulations in the induction of γδ T cell responses and the consequent cascade of events.
3. To identify the transcriptomic signature of brain-infiltrating DCs.
4. To investigate the chemoattractant factors that drive the infiltration of DC subsets to the ischaemic brain.
5. To investigate the effect of DC subpopulations on the outcome of ischaemic stroke.

Results

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Barcelona, 14th December 2020

REPORT OF THE SUPERVISORS OF THE Ph.D. THESIS OF Mr. MATTIA GALLIZIOLI

As directors of this thesis, we hereby declare that the student MATTIA GALLIZIOLI presents his PhD thesis in the form of compendium of publications. The thesis has two papers published in peer-reviewed international scientific journals. The two publications are the result of his Ph.D. work, he has taken a leading part in the development of the study and has participated in the design, performance of the study, interpretation, and discussion.

ARTICLE #1
Gelderblom, M.*, Gallizioli, M.*, et al. (2018). IL-23 (Interleukin-23)–Producing Conventional Dendritic Cells Control the Detrimental IL-17 (Interleukin-17) Response in Stroke.
Stroke 49, 155–164 DOI: 10.1161/STROKEAHA.117.019101. (* equal contribution as 1st authors)
Impact factor 2019-2020: 5.440 D1
According to the CRediT taxonomy of contributor roles (Brand et al., 2015), in this article Mattia Gallizioli contributed significantly to: Conceptualization, Methodology, Formal Analysis, Investigation, Writing – Review & Editing, Visualization. Mattia Gallizioli is co-first author since he shared with Dr. Gelderblom the work development. He was involved in most experiments and took a leading part on them.

ARTICLE #2
Gallizioli, M., et al. (2020). Dendritic Cells and Microglia Have Non-redundant Functions in the Inflamed Brain with Protective Effects of Type 1 cDCs.
Cell Reports 33, 108291 DOI: 10.1016/j.celrep.2020.108291.
Impact factor 2019-2020: 7.700 Q1
According to the CRediT taxonomy of contributor roles (Brand et al., 2015), in this article Mattia Gallizioli contributed significantly to: Conceptualization, Methodology, Formal Analysis, Investigation, Writing – Review & Editing, Visualization. His deserved the role of 1st author since this work is a main part of his Ph.D. project. He conducted most of the experimental animal work, flow cytometry, MRI and transcriptomics, and co-leaded the development of the project together with the directors.

These articles were not previously presented and will not be presented in any other PhD thesis.

The directors,

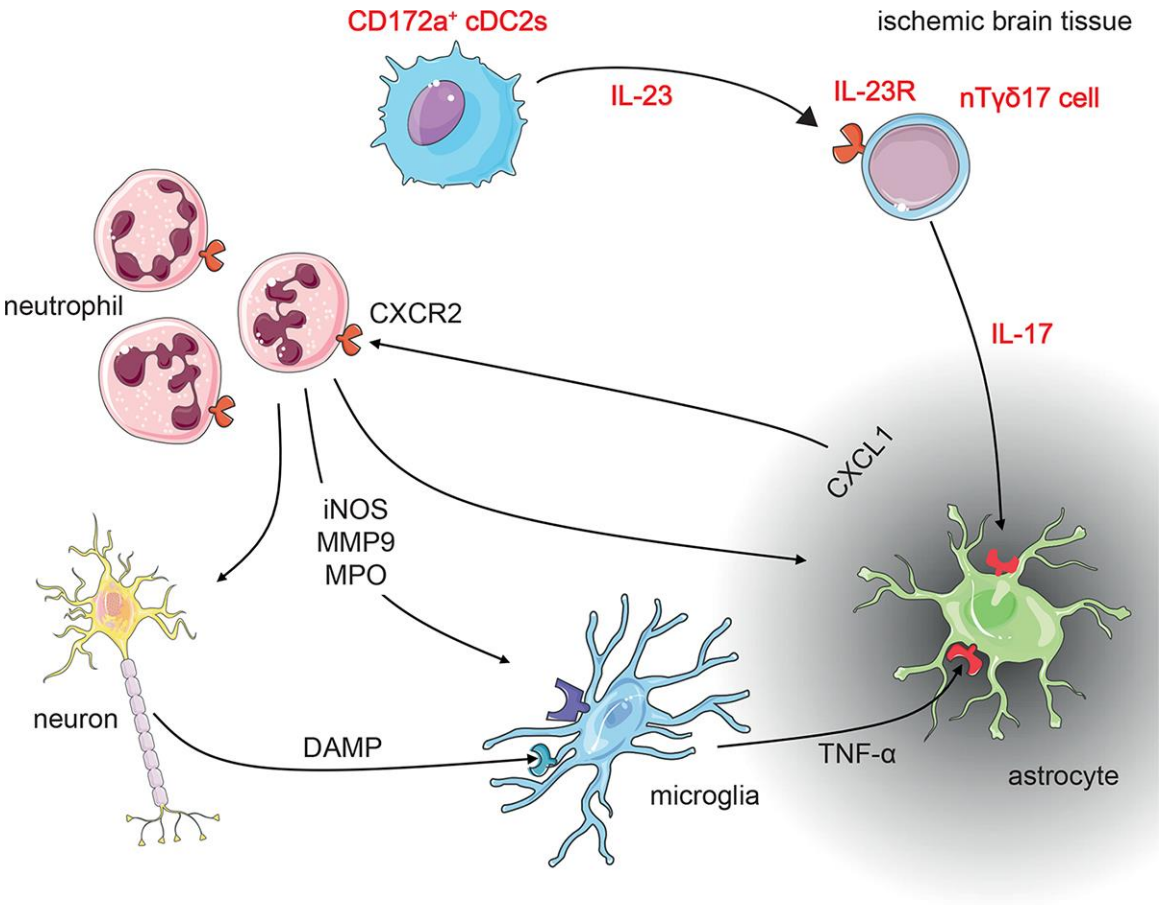
Ana Maria Planas Obradors Francesc Miró Mur

SUMMARY OF RESULTS

The results obtained are resumed here and presented in the form of compendium of publications.

SUMMARY OF ARTICLE #1

Gelderblom, M.*, **Gallizioli, M.***, et al. (2018). IL-23 (Interleukin-23)–producing conventional dendritic cells control the detrimental IL-17 (Interleukin-17) response in stroke. *Stroke* 49, 155–164. DOI: 10.1161/STROKEAHA.117.019101
** indicates equal contribution*



Graphical abstract from Gelderblom et al., 2018

In this paper, we studied the involvement of cDC2 dendritic cells in the early response to transient middle cerebral artery occlusion in mice.

We first confirmed previous results showing that CD11c$^+$ cells infiltrate the mouse brain from 24h to 7 days after stroke (Felger et al., 2010) by immunofluorescence of CD11c-eYFP animals. We then developed a flow cytometry panel to study the relative contribution of DC subpopulations in this infiltration. With this analysis we demonstrated that the main subpopulation infiltrating the brain 24h after stroke was the conventional DCs, which peaked at day 3 after stroke, to then decline at day 7. We then further divided the conventional DCs into cDC1 and cDC2, thanks to the expression of XCR1 and CD172a respectively. In this way we observed that the majority of infiltrating cDC were of the type 2 subpopulation, with a smaller contribution by cDC1 starting at day 1 after ischaemia. By FACS, we isolated the cDC2 subpopulation and verified its identity by observing high expression level of *Zbtb46* and *Irf4* and low levels of *Irf8* and *Batf3*, a transcriptional signature typical of cDC2 cells.

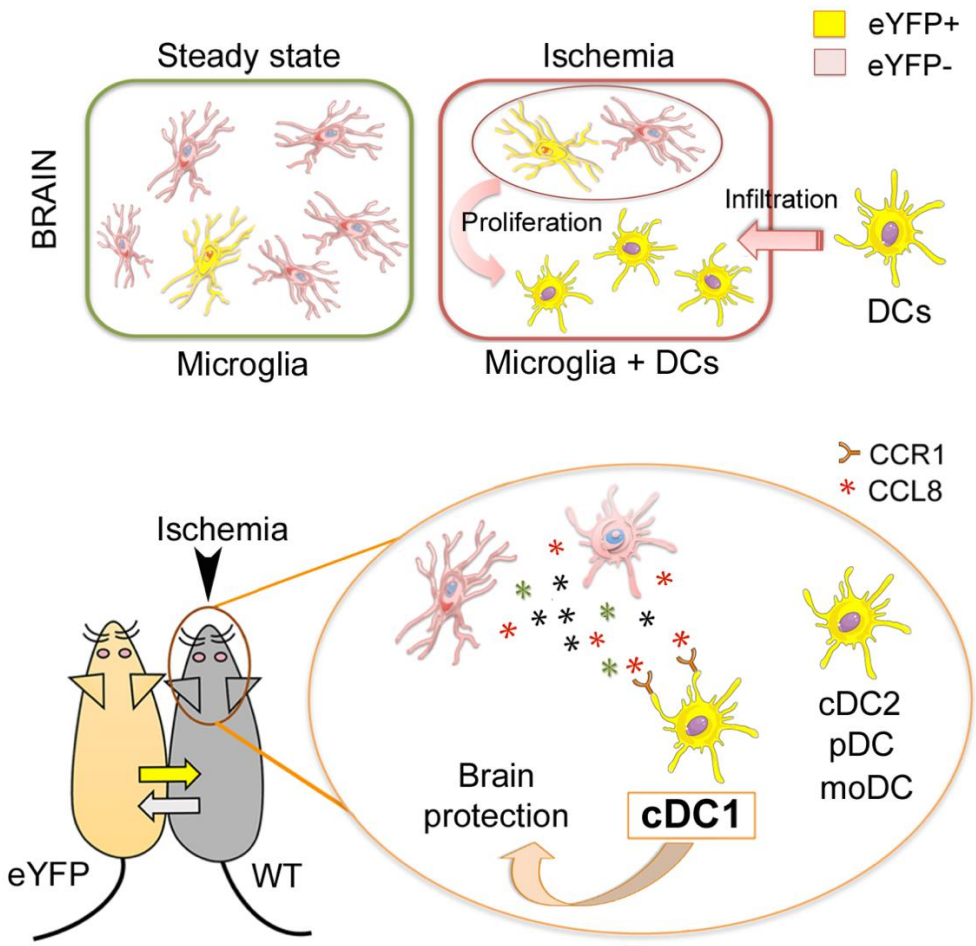
We administered diphtheria toxin (DTX) to CD11c.DOG mice, in which a diphtheria toxin receptor is expressed in CD11c$^+$ cells, to deplete DCs. The animals treated with the toxin showed reduced infarct volume, better neurological outcome and reduced mortality compared with toxin-treated wild type littermates. Furthermore, we observed a reduction of neutrophil infiltration at days 1 and 3 after stroke.
Since it had been previously shown that neutrophil infiltration into the ischaemic brain is driven by CXCL-1 produced by astrocytes and that these cells were stimulated by IL-17 produced by γδ T cells, we investigated how this axis was affected by the depletion of CD11c$^+$ cells.
After CD11c$^+$ cell depletion we observed a significant reduction of IL-17 levels in brain-infiltrating γδ T cells, while no alterations were evident in other classical T cell subsets. We also observed a parallel reduction of *Cxcl1* RNA expression in the brain tissue of DTX-treated CD11c.DOG mice, highlighting the disruption of the IL-17-CXCL1 axis in the brain of these mice.
Given that IL-23 has been shown to regulate IL-17 production (Croxford et al., 2012) and that we found that approximately 80% of the brain-infiltrating IL-17$^+$ γδ T cells expressed the IL-23 receptor, we analysed the production of IL-17 by γδ T cells in the brain of homozygous *Il23r.gfp.KI* (*Il23r*$^{-/-}$) mice, which do not express a functional IL-23 receptor.
In the ischaemic brain of these mice the frequency of IL-17$^+$ γδ T cells and the expression of *Cxcl1* was significantly reduced, ultimately leading to a sharp reduction of neutrophil infiltration. Importantly, infarct size and neurological deficits were also reduced in *Il23r*$^{-/-}$ mice.

To discern the contribution of IL-23 receptor specifically expressed on T cells, we adoptively transferred T cells from *Il23r*$^{-/-}$ mice or wild type littermates into *Rag1*$^{-/-}$ mice, which are constitutively lacking T cells. The infiltration of neutrophils in the ischaemic brain was again reduced in mice that received *Il23r*$^{-/-}$ T cells, compared with mice that received wild type T cells. Since cDC2 were the most prominent infiltrating subpopulation in the ischaemic brain, we measured the production of *Il23p19* and *Tnf* transcripts in sorted cDC2, macrophages and microglia. *Il23p19* transcript expression was significantly upregulated in cDC2 compared with both macrophages and microglia. Furthermore, brain cDC2 displayed a significant increase in the expression of this transcript also when compared with splenic cDC2, indicating a specific induction of the expression in the brain. Consistent with this result, the level of *Il23p19* transcript was significantly reduced in the ischaemic brain of DTX-treated CD11c.DOG mice.
To further confirm that the main source of IL-23 were the infiltrating peripheral cDC2, we generated bone marrow chimeras and observed that irradiated wild type animals reconstituted with *Il23p19*$^{-/-}$ bone marrow displayed reduced neutrophil infiltration into the ischaemic hemisphere.

In brief, this study showed the negative role of cDC2 in the ischaemic brain tissue, where these cells are involved in the activation of γδ T cells and the recruitment of neutrophils.

SUMMARY OF ARTICLE #2

Gallizioli, M., et al. (2020). Dendritic cells and microglia have non-redundant functions in the inflamed brain with protective effects of type 1 cDCs. *Cell Reports* 33, 108291. DOI: 10.1016/j.celrep.2020.108291



Graphical abstract from Gallizioli et al., 2020

In this paper, we identified several peculiarities of brain infiltrating dendritic cells when compared with resident microglia. Furthermore, we observed that microglia attract dendritic cells to the ischaemic brain, remarkably cDC1, which appear to have a beneficial role in cerebral ischaemia.

We first confirmed infiltration of CD11c^{+} cells to the ischaemic brain by immunofluorescence of CD11c-eYFP mice, which express eYFP under the control of the CD11c promoter. To find out whether this increase in the brain after stroke was dependent on FLT3L, we administered it to mice systemically for 7 days, leading to

increased number of CD11c$^+$ cells in the spleen and in the brain 4 days after ischaemia. In the brain, astrocytes were the most potent producers of *Flt3lg*, whose amount increased after stroke.
Even though CD11c$^+$ cells shared morphological and phenotypical markers with microglia, the former showed lower expression of typical microglial genes, indicating peculiar characteristics. To further investigate the difference between CD11c-eYFP cells and microglia, we performed RNA-Seq analysis of FACS-sorted cells from ischaemic and control brains. The analysed portion of the transcriptome of brain eYFP$^+$ cells, albeit being clearly different from microglia, was not strikingly similar to any of the ImmGen populations we chose for the comparison, indicating that brain eYFP$^+$ cells may have tissue-specific features.

We then performed a functional comparison of CD11c$^+$ cells and microglia, with special interest in antigen presentation capacity. RNA-Seq data indicated overexpression of MHCII-related genes in eYFP$^+$ cells compared with microglia in the ischaemic brain. Similarly, genes encoding immunomodulatory and costimulatory molecules necessary for antigen presentation were more expressed in eYFP$^+$ cells. For a functional assessment of antigen presentation, we co-cultured ischaemic microglia or infiltrating eYFP$^+$ cells both with OTII and OTI CD4$^+$ T. In both cases infiltrating eYFP$^+$ cells were the most efficient at inducing antigen-dependent T cell proliferation. Altogether these data indicate that infiltrating eYFP$^+$ cells can be considered a bona fide DC population.

In order to unequivocally distinguish infiltrating from brain-derived CD11c-eYFP cells and to study their migration form the periphery, we generated parabiotic (PA) pairs by joining the circulation of wild type and CD11c-eYFP littermates.
In steady-state conditions eYFP$^+$ cells were mainly located in the leptomeningeal spaces and in the choroid plexus, not in the brain parenchyma.
Four days after inducing ischaemia in the wild type parabiont, we observed eYFP$^+$ cells in the brain parenchyma, leptomeninges and choroid plexus, indicating active infiltration upon ischaemia.
We compared the transcriptomic profile of brain infiltrating eYFP$^+$ cells FACS-sorted from the ischaemic brain of the wild type parabiont mice with that of microglia from ischaemic mice and total brain eYFP$^+$ cells from ischaemic CD11c-eYFP non-parabiotic mice. PA eYFP$^+$ cells showed increased expression of various C-type lectin receptors when compared with microglia, possibly indicating some degree of cellular specialization in the sensing and processing of innate immune signals. Gene set enrichment analysis of these data identified overrepresentation of

cell cycle-related genes in microglia, compatible with a more proliferative phenotype of these cells compared with infiltrating eYFP$^+$ cells.

RNA-Seq data and RT-PCR analysis of independent samples showed increased expression of various chemokine receptors in eYFP$^+$ cells compared with microglia, most notably *Ccr1*, *Ccr2* and *Ccr7*. We also observed increased expression of several chemokines that bind to those receptors. We were able to identify microglia as the main producer of *Ccl5* and *Ccl8*, potential attractants for DCs.

We therefore depleted microglia by administering the CSF1R inhibitor PLX5622. The treatment reduced the number of infiltrating DCs, while leaving the peripheral population of DCs unaltered; the effect was directly attributable to the reduction of chemokine production in the ischaemic brain tissue after microglia depletion. Notably, the subpopulation of DCs most affected by microglia depletion was the cDC1 subset.

By blocking CCR1 with the antagonist J113863 we were able to significantly reduce the infiltration of cDC1s to the ischaemic brain. The animals that received the drug had a worse evolution of the neurological outcome from day 1 to day 4 compared with vehicle-treated mice, suggesting beneficial effects of cDC1 in brain ischaemia.

Batf3$^{-/-}$ mice, which do not develop cDC1 cells, also showed larger infarcts and worse neurological outcome than WT littermates. In chimeric WT animals reconstituted with *Batf3*$^{-/-}$ bone marrow we observed increased infarct volumes from day 1 to day 4 and worse neurological deficit at day 4 after ischaemia, when compared to WT-reconstituted mice, confirming the role of peripheral infiltrating cDC1.
Altogether these results indicate a beneficial role of *Batf3*-dependent cDC1 cells in brain ischaemia.

To resume, in this study we showed that the CD11c$^+$ population of cells present in the brain after ischaemia is composed of proliferating microglia and infiltrating dendritic cells, which exhibit a peculiar transcriptional profile and excel in antigen presentation. We also demonstrate that microglia can attract dendritic cells by producing chemokines, with a potent effect on the infiltration of cDC1, which play a beneficial role in the ischaemic brain.

Basic Sciences

IL-23 (Interleukin-23)–Producing Conventional Dendritic Cells Control the Detrimental IL-17 (Interleukin-17) Response in Stroke

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Background and Purpose—Inflammatory mechanisms can exacerbate ischemic tissue damage and worsen clinical outcome in patients with stroke. Both αβ and γδ T cells are established mediators of tissue damage in stroke, and the role of dendritic cells (DCs) in inducing the early events of T cell activation and differentiation in stroke is not well understood.

Methods—In a murine model of experimental stroke, we defined the immune phenotype of infiltrating DC subsets based on flow cytometry of surface markers, the expression of ontogenetic markers, and cytokine levels. We used conditional DC depletion, bone marrow chimeric mice, and IL-23 (interleukin-23) receptor-deficient mice to further explore the functional role of DCs.

Results—We show that the ischemic brain was rapidly infiltrated by IRF4$^+$/CD172a$^+$ conventional type 2 DCs and that conventional type 2 DCs were the most abundant subset in comparison with all other DC subsets. Twenty-four hours after ischemia onset, conventional type 2 DCs became the major source of IL-23, promoting neutrophil infiltration by induction of IL-17 (interleukin-17) in γδ T cells. Functionally, the depletion of CD11c$^+$ cells or the genetic disruption of the IL-23 signaling abrogated both IL-17 production in γδ T cells and neutrophil infiltration. Interruption of the IL-23/IL-17 cascade decreased infarct size and improved neurological outcome after stroke.

Conclusions—Our results suggest a central role for interferon regulatory factor 4-positive IL-23–producing conventional DCs in the IL-17–dependent secondary tissue damage in stroke.

Visual Overview—An online visual overview is available for this article. **(*Stroke*. 2018;49:155-164. DOI: 10.1161/STROKEAHA.117.019101.)**

Key Words: dendritic cells ■ inflammation ■ interleukin-17 ■ interleukin-23 ■ stroke

Ischemic stroke is the primary cause for sustained disability in the Western world. The initial ischemic brain damage leads to a robust activation of the immune system, which follows a pattern typical of sterile inflammation. Ischemic tissue hallmarks are the presence of IL-17 (interleukin-17)–positive γδ T cells, secondary neutrophil infiltration, the appearance of monocytes, and an upregulation of proinflammatory cytokines and chemokines.[1–3] Even though effector functions of γδ T cells have been mostly decoded, the triggering inflammatory events are largely unclear.[2,4]

Recently, it has been shown that apart from their role in classical concepts of antigen-dependent immune responses,[5] dendritic cells (DCs) are capable of locally shaping immune reactions independently of their migration to secondary lymphoid organs or antigen presentation.[6] Engagement of toll-like receptors on DCs by pathogen-associated molecular patterns or danger-associated molecular patterns lead to the rapid production of cytokines, which in turn can start a fast innate-like immune cascade. For example, both conventional DCs (cDCs) and

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monocyte-derived DCs can produce IL-23 (interleukin-23) upon stimulation with TLR5 ligands.[7]

In cerebral ischemia, a significant amount of CD11c$^+$ cells appears early in the disease course.[8,9] However, the origin, phenotype and function of these cells are still unknown. Because of the exquisite ability of DCs to mount immune responses, these CD11c$^+$ cells could be of importance for the inflammatory response after stroke.

The identification and classification of these cells in the brain is of critical importance and has been recently debated.[10] In this study, we have used highly detailed multiparameter flow cytometry to identify brain-infiltrating DC subsets according to the classification recently proposed by Guilliams et al,[11] namely conventional type 1 DCs (cDC1s), conventional type 2 DCs (cDC2s), and plasmacytoid DCs. We further differentiated cDCs and plasmacytoid DCs from monocyte-derived DCs.

Using this approach, we identified in the ischemic hemisphere the presence of conventional CD172a$^+$/IRF4$^+$ type 2 DCs that were the major source of IL-23, which is essential for the expression of IL-17 in γδ T cells.

Materials and Methods

The data that support the findings of this study are available from the corresponding author on reasonable request.

Animals

All animal experiments were approved by local animal care committees (Behörde für Lebensmittelsicherheit und Veterinärwesen Hamburg). We conducted the experiments according to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (publication No. 83–123, revised 1996) and performed all procedures in accordance with the ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments; http://www.nc3rs.org/ARRIVE). We randomized all mice and conducted transient middle cerebral artery occlusion (tMCAO) as described previously.[2] The detailed experimental description can be found in the online-only Data Supplement.

Analysis of Infarct Size by TTC Staining and Magnetic Resonance Imaging

We analyzed infarct size by vital staining using 2% (wt/vol) TTC (2,3,5-triphenyl-2-hydroxy-tetrazolium chloride) in phosphate buffer. Magnetic resonance imaging was performed on a dedicated 7T MR small animal imaging system (ClinScan; Bruker). Detailed experimental description can be found in the online-only Data Supplement.

Antibodies and Flow Cytometry

We performed flow cytometry for the analysis of cell types as described previously.[2] Detailed experimental description can be found in the online-only Data Supplement.

Cell Sorting, RNA Isolation, and Quantitative Real-Time Polymerase Chain Reaction

Immune cells were sorted using a BD fluorescence-activated cell sorting Aria IIIu, and quantitative real-time polymerase chain reaction was performed as described previously.[2] Detailed experimental description can be found in the online-only Data Supplement.

Immunohistochemistry and Immunofluorescence

We stained mouse brains with antibodies against Ly6G (1A8; Biolegend), GFAP (glial fibrillary acidic protein; Dako), Iba-1 (Wako), and laminin (1:100, Dako). Autoptic human brain sections were stained with antibodies against CD11c (Abcam, ab52632) and CD11b (Abcam, ab52478). Detailed experimental description can be found in the online-only Data Supplement.

Cell Transfer

We performed cell transfers as described previously.[2]

Bone Marrow Chimeras

Recipients were irradiated by whole-body irradiation (9 Gy) using a cesium-137 gamma irradiator (BIOBEAM 2000). Detailed experimental description can be found in the online-only Data Supplement.

Statistical Analysis

Statistical analyses were performed using the appropriate test indicated in the figure legends. Briefly, we used Student *t* test to compare infarct volumes, Mann–Whitney *U* test for the comparison of clinical scores, 1-way ANOVA for multiple comparisons with Bonferroni post hoc test, after validating the normal distribution of the data sets (Kolmogorov–Smirnov test), and 1-way ANOVA with Bonferroni post hoc test and unpaired Student *t* test for the comparison of ΔΔCt values between groups. P values <0.05 were considered statistically significant.

Results

DCs Infiltrate the Brain Parenchyma Early and Locate to the Perilesional Area

The number of CD11c$^+$ DCs was low in the healthy brain. However, after tMCAO, CD11c$^+$ DCs had entered the ischemic brain and settled already at 24 hours to the peri-infarct area and, more specifically, close to blood vessels (Figure 1A). Next, we examined by immunohistochemistry postmortem stroke tissue from patients who had died shortly after having a stroke (<24 hours; Figure 1B). While we rarely observed CD11c$^+$ cells in normal brain tissue, the CD11c expression increased in the penumbra area after stroke. The different expression patterns of CD11c and CD11b in postmortem stroke tissue indicate that the upregulation of CD11c is not only because of activation of CD11b$^+$ microglia but also because of a consequence of infiltrating CD11c$^+$ immune cells. To further analyze which DC subsets infiltrate the brain in our experimental stroke model, we used whole-brain fluorescence-activated cell sorting analysis. In a first step, DCs were classified as CD45^{high}CD11c$^+$MHCII$^+$Ly6G$^-$, thus excluding the CD45^{int}-expressing microglia and the Ly6G$^+$ neutrophils. Inside this population, we differentiated plasmacytoid DCs as B220$^+$, cDCs as B220$^-$Ly6C$^-$CD64$^-$F4/80^{low}, and monocyte-derived DCs as Ly6C^{int}F4/80^{int}SSC^{int}CD64^{int} (Figure 1C).[11] cDCs were the most prevalent DC subset at all time points. Already after 24 hours, we observed a significant increase to 4414±3329 cDCs per hemisphere when compared with 339±90 in sham-operated animals. cDCs reached maximum levels at day 3 with a subsequent decrease at day 7.

CD172a$^+$/*Irf4*-Expressing cDC2 Cells Are the Predominant Subpopulation of Brain-Infiltrating cDCs

In nonlymphoid tissues, cDCs can further be differentiated into XCR1$^+$/IRF8$^+$ cDC1s and CD172a$^+$/IRF4$^+$ cDC2s.[12] Flow cytometric analysis revealed that the vast majority of infiltrating cDCs was XCR1$^-$/CD172a$^+$ cDC2s at all investigated time points (Figure 2A). We also observed CD172a$^-$/XCR1$^+$ cDC1s,

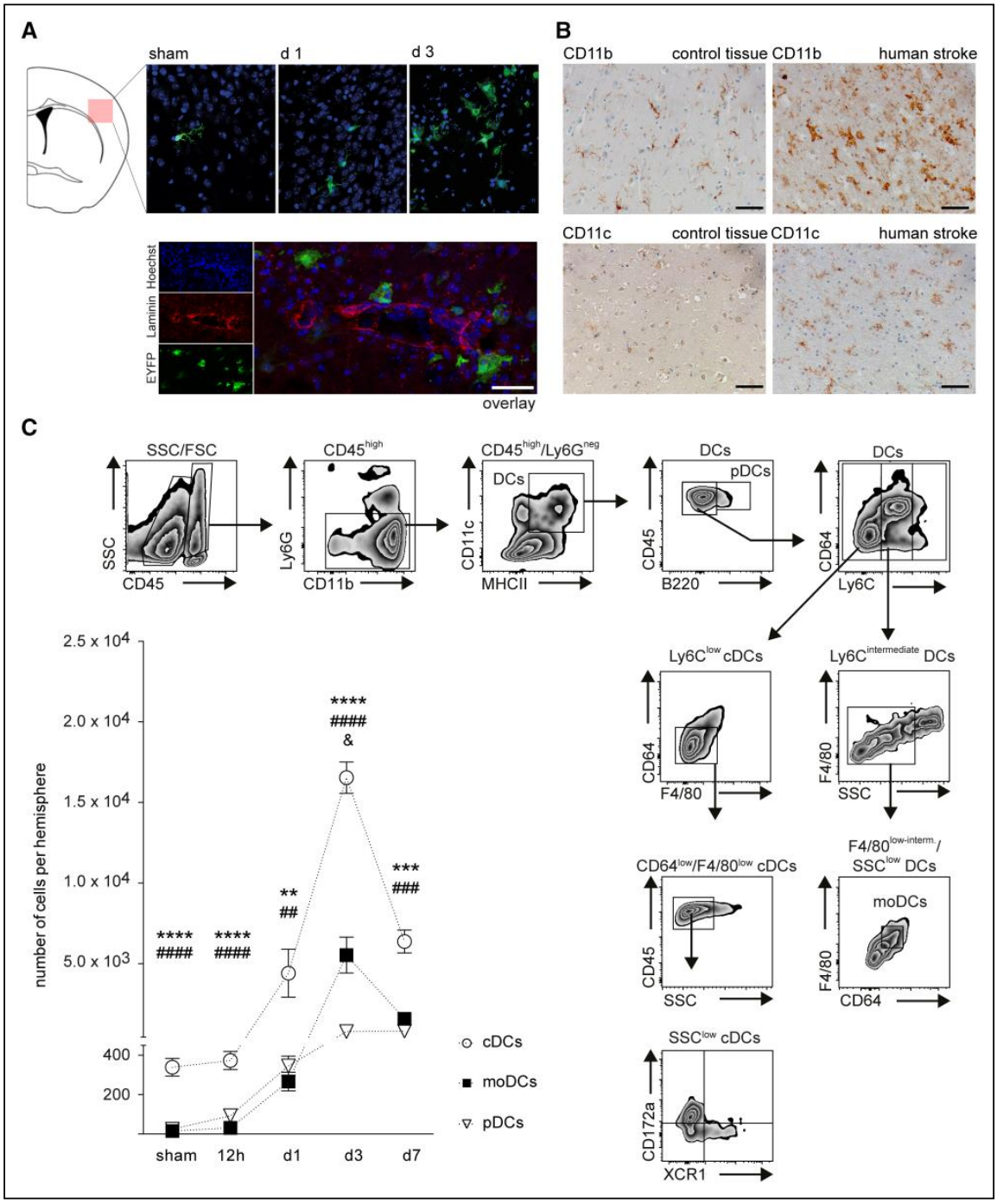

Figure 1. Dendritic cells (DCs) infiltrate the ischemic hemisphere and locate to the penumbra area. **A**, Visualization of EYFP (enhanced yellow fluorescent protein)-positive DCs in the penumbra of ischemic hemispheres of *Cd11c*-EYFP mice 1 and 3 days after transient middle cerebral artery occlusion (scale bars, 20 µm), and histological staining for laminin in ischemic hemispheres of *CD11c*-EYFP-mice at day 3 (scale bar, 0.7 mm). **B**, Immunohistochemical analysis of immune cells in ischemic human brain tissue 24 hours after stroke and unaffected brain tissue. Analysis was performed to detect CD11b and CD11c (scale bars, 30 µm). **C**, Flow cytometry of brain-infiltrating DC subsets. Gating strategy to identify plasmacytoid DCs (pDCs), monocyte-derived DCs (moDCs), and conventional DCs (cDCs) after staining for B220, CD11b, CD11c, CD45, CD64, CD172a, F4/80, Ly6C, Ly6G, MHCII, and XCR1. For absolute quantification, TrueCount tubes were used. **C**, The graphs show mean±SD of 4 to 6 animals per group, in 3 to 5 independent experiments for each time point. Statistical significances analyzed by 1-way ANOVA with Bonferroni post hoc test. $^{**}P<0.01$. FSC indicates forward scatter; and SSC, side scatter. *cDCs vs moDCs, #cDCs vs pDCs, and moDCs vs pDCs.

which exhibited delayed infiltration kinetics. To validate that the XCR1⁻/CD172a⁺ cDCs found in the brain belong to the cDC2 subset, we relied on expression levels of C-C motif chemokine receptor 2 (CCR2) and ontogenetic markers. As reported previously,[13] we also found that cDC2s had a CCR2^{high} profile, whereas microglia showed significantly lower CCR2 levels (Figure 2B). We next analyzed the expression of established transcription factors in fluorescence-activated cell-sorted

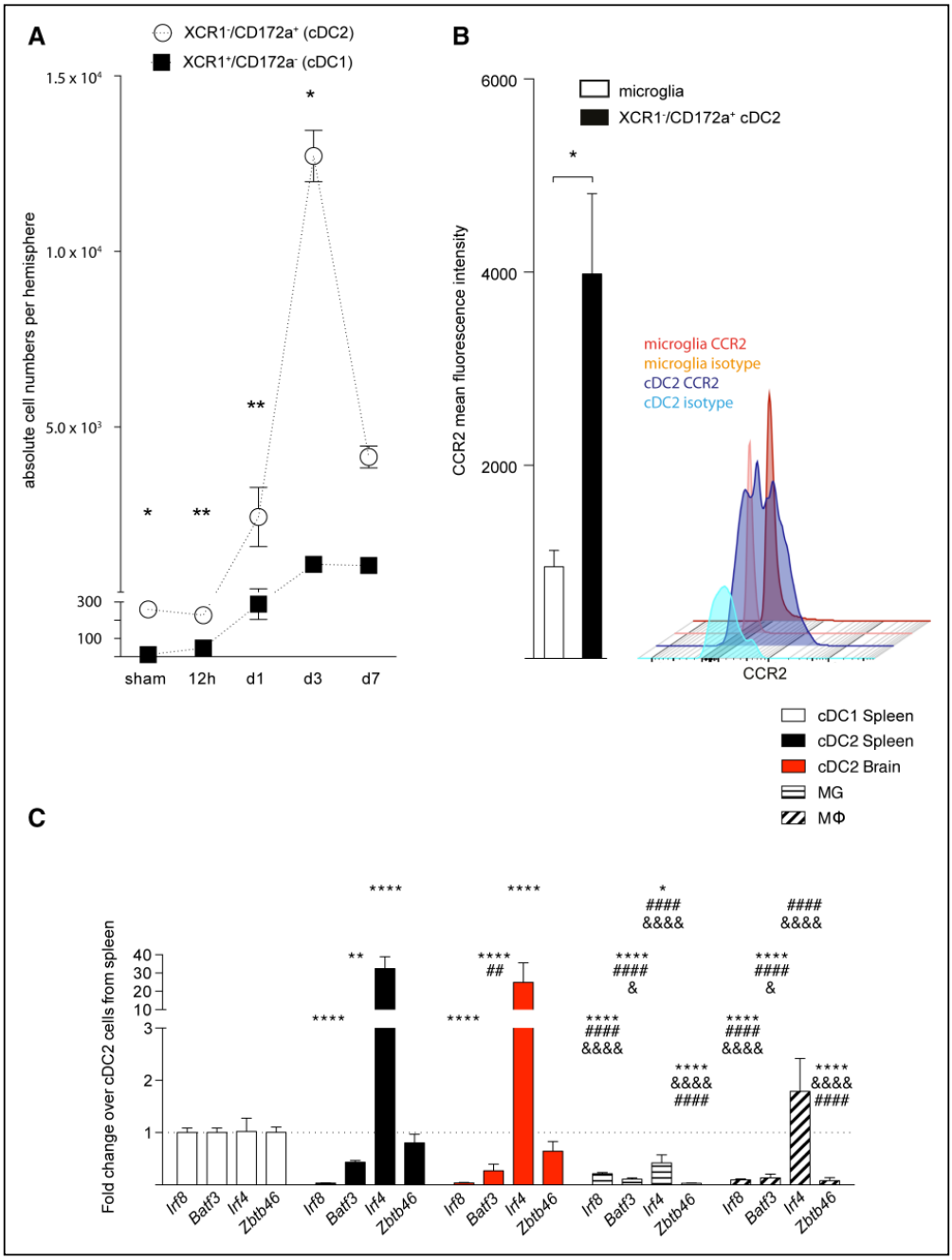

Figure 2. Classification of infiltrating dendritic cell subsets by immunophenotyping and analysis of ontogenetic markers. **A**, Absolute numbers of XCR1⁺ cDC1s and CD172a⁺ conventional type 2 DCs (cDC2s) in ischemic hemispheres after transient middle cerebral artery occlusion (tMCAO). Cell counts were determined by flow cytometric analysis of central nervous system-infiltrating cells after Percoll density centrifugation. For absolute quantification, TrueCount tubes were used. **B**, Flow cytometric analysis of CCR2 level on central nervous system-infiltrating CD172a⁺ cDC2s and microglia 24 hours after tMCAO. **C**, Relative gene expression of *Irf4*, *Irf8*, *Batf3*, and *Zbtb46* in central nervous system-infiltrating and splenic CD45^{high}/CD11b⁺/CD11c⁺/MHCII⁺/CD172a⁺/Ly6c⁻/Ly6g⁻/F4/80⁻/XCR1⁻ cDC2, CD45^{intermed}/CD11b⁺ microglia and central nervous system-infiltrating CD45^{high}/CD11b⁺/CD11c⁻/MHCII⁻/Ly6g⁻/F4/80⁺ macrophages purified 24 hours after tMCAO by fluorescence-activated cell sorting. Expression levels were normalized to corresponding levels of splenic cDC1s. The graphs show mean±SEM of 5 to 8 animals per group, in 5 to 7 independent experiments for each time point. Statistical significances analyzed by (**A**) 2-way ANOVA with Bonferroni post hoc test, (**B**) Student *t* test, and (**C**) 1-way ANOVA with Bonferroni post hoc test. **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001. *vs cDC1 spleen, #vs cDC2 spleen, and vs cDC2 brain. MΦ indicates macrophages; and MG, microglia.

cDC2s from ischemic hemispheres at 12 and 24 hours after tMCAO. After normalization to corresponding levels in splenic cDC1s, we observed high levels for *Irf4* in combination with low levels for *Irf8* and *Batf3* in CD172a⁺ cDC2s derived from ischemic brains and spleens (Figure 2C; Figures I and II in the online-only Data Supplement). Furthermore, the expression of the DC-specific gene *Zbtb46* was maintained in cDC2s. Overall, high levels for *Irf4*, low levels for *Irf8* and *Batf3*, and expression of CD172a and CCR2 are consistent with the established profile of cDC2s. Most importantly, microglia and

macrophages extracted from same ischemic hemispheres displayed substantially different gene expression profiles. The results demonstrate the abundance of the newly described cDC2 cell population in the ischemic brain tissue.

Depletion of CD11c⁺ Cells in the *Cd11c.DOG* Model Is Protective in Stroke

Next, we used the *Cd11c.DOG* mouse model, in which a DTR (diphtheria toxin receptor) is expressed under the CD11c promoter.[14] The *Cd11c.DOG* model is widely used to deplete conventional CD11c⁺ DCs via injection of diphtheria toxin (DTX).[15] To control potential toxic effects of DTX, we treated heterozygous *Cd11c.DOG* mice and littermate controls with DTX. Daily injections of 8 ng/g body weight DTX started 24 hours before the tMCAO procedure[14] and resulted in a significant reduction of splenic and brain-infiltrating DCs (Figure 3A and 3B). Interestingly, DTX-treated *Cd11c.DOG* mice showed significantly reduced infarct sizes, milder neurological symptoms, and reduced mortality compared with DTX-treated littermate controls (Figure 3C and 3D).

CD11c⁺ Cells Induce Neutrophil Infiltration Into Ischemic Hemispheres Via Induction of IL-17 in γδ T Cells

Analysis of the immune cell infiltration in ischemic brains of *Cd11c.DOG* mice revealed significant reduction in neutrophil infiltration at days 1 and 3. Absolute numbers of other infiltrating leukocyte populations were not significantly affected (Figure 4A and 4B; Figure IIIA in the online-only Data Supplement). In fact, we saw an increase in neutrophils in

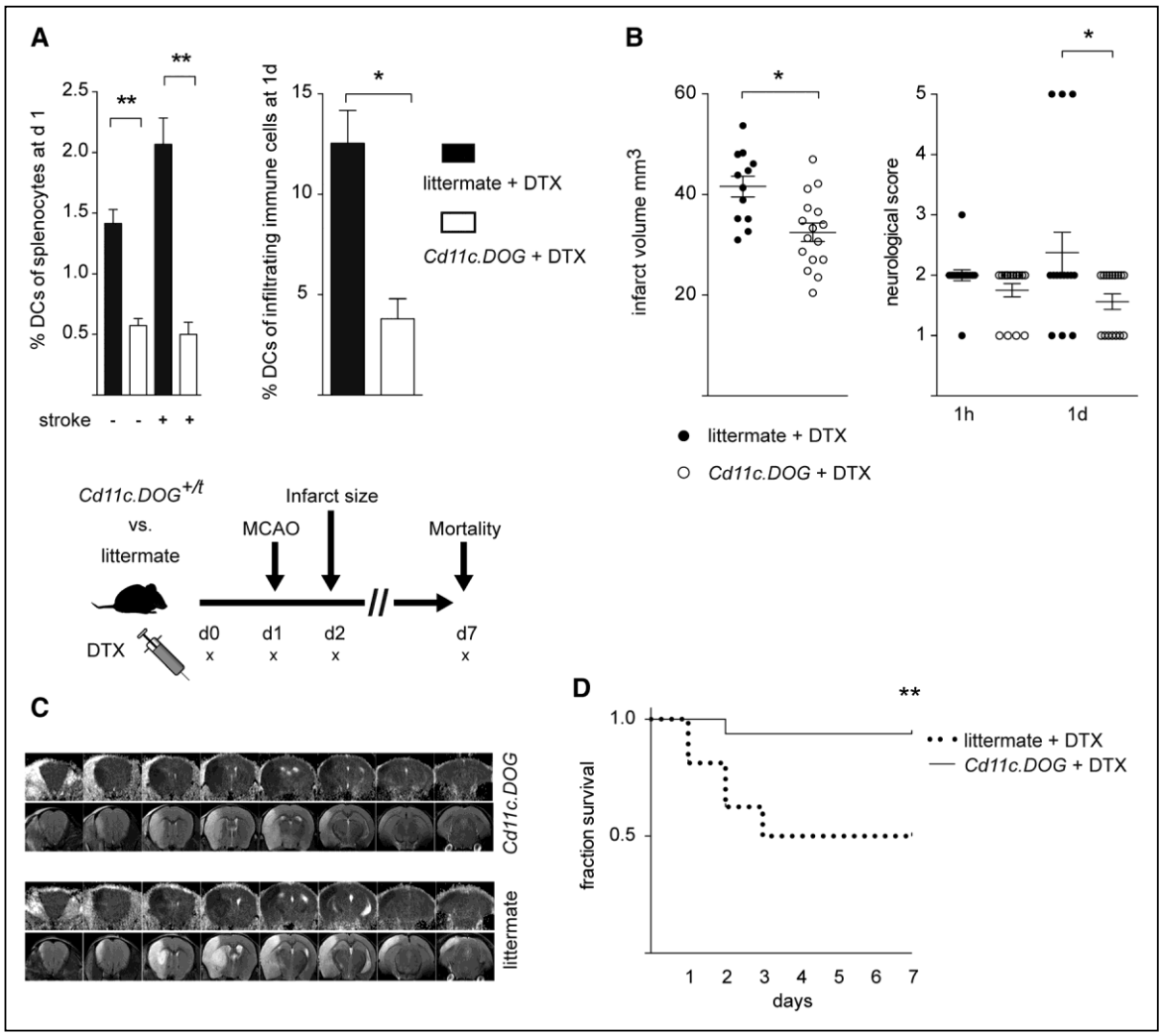

Figure 3. Depletion of CD11c⁺ cells in *Cd11c.DOG* mice is protective in a murine model of ischemic stroke. **A**, Depletion of CD11c⁺ cells in *Cd11c.DOG* mice after continuous daily administration of 8 ng/g body weight diphtheria toxin (DTX). Graph shows the percentages of splenic CD11c⁺/MHCII⁺ dendritic cells (DCs) of total living splenocytes in *Cd11c.DOG* mice compared with littermate controls 3 days after DTX administration. Infiltration of CD11c⁺/MHCII⁺ cells into ischemic hemispheres in *Cd11c.DOG* mice and littermate controls 1 day after transient middle cerebral artery occlusion (tMCAO). Daily administration of DTX was started 1 day before tMCAO. Cell counts were performed by flow cytometric analysis of splenocytes and central nervous system-infiltrating cells after staining for CD11b, CD11c, CD45, Ly6G, and MHCII. Graphs show the mean±SD of 9 to 12 animals per group from 3 to 4 independent experiments. **B** and **C**, Magnetic resonance imaging was used to quantify (representative T2 image) infarct volume at day 1 after tMCAO (**left**) in littermate controls and *Cd11c.DOG* mice after daily DTX administration. Neurological scores were performed 1 hour and 1 day (**right**) after middle cerebral artery occlusion (MCAO). Data are presented as mean±SD of 12 littermate controls and 16 *Cd11c.DOG* animals. Statistical significances analyzed by (**A** and **B**) Student *t* test, Mann–Whitney *U* test (neurological scores), and (**D**) χ^2 test (survival rate). **P*<0.05, ***P*<0.01.

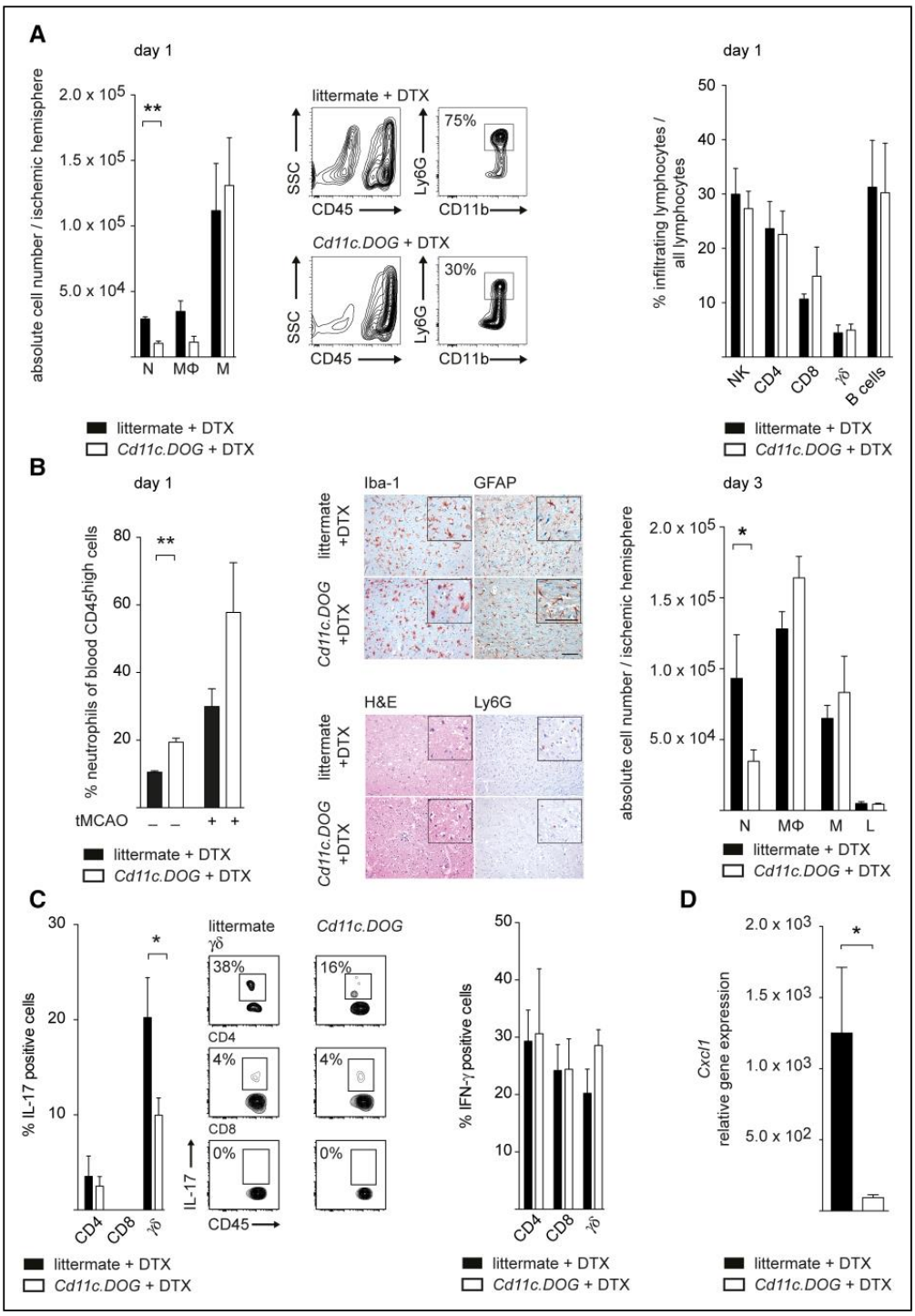

Figure 4. Depletion of CD11c$^+$ cells alters the migration of neutrophils and the production of IL-17 (interleukin-17) by γδ T cells. **A**, Absolute numbers of neutrophils (n; CD45^{high}/Ly6G$^+$/CD11b$^+$), macrophages (mΦ; CD45^{high}/Ly6G$^-$/CD11b$^+$), microglia (m; CD45^{intermediate}/CD11b$^+$), and frequency of CD4$^+$, CD8$^+$, γδ T cells, NK cells, and B cells in ischemic hemispheres of diphtheria toxin (DTX)-treated littermate controls and *Cd11c.DOG* mice 1 day after middle cerebral artery occlusion (MCAO). **B**, Frequency of peripheral blood neutrophils after DTX administration in littermate controls and *CD11c.DOG* mice 1 day after MCAO or sham surgery, immunohistochemical staining of macrophages/microglia (Iba-1), astrocytes (GFAP [glial fibrillary acidic protein]), and neutrophils (Ly6G) in DTX-treated littermate controls and *CD11c.DOG* mice 1 day after MCAO (scale bar, 50 μm), and absolute numbers of neutrophils (N), macrophages (MΦ), microglia (M), and lymphocytes (L) 3 days after MCAO. **C**, Flow cytometric analysis of IL-17 (interleukin-17) and IFN-γ (interferon-γ) produced by CD4$^+$, CD8$^+$, and γδ T cells isolated from ischemic hemispheres at day 1 after stroke induction. **A–C**, Cell counts were determined by flow cytometric analysis. **D**, Relative gene expression of *Cxcl1* in ischemic hemispheres of DTX-treated littermate controls and *Cd11c.DOG* mice 24 hours after transient middle cerebral artery occlusion (tMCAO). **A–D**, Daily DTX administration of 8 ng/g body weight DTX was started 1 day before MCAO or sham surgery. Data show the mean±SD of 9 to 12 animals per group, analyzed in 3 to 4 (**A–C**) or 6 (**D**) independent experiments. Statistical significances were analyzed by Student *t* test in all cases. *$P<0.05$, **$P<0.01$. SSC indicates side scatter.

the peripheral blood of *Cd11c.DOG* mice treated with DTX (Figure 4B), which further underscores the dysfunctional neutrophil migration into the brain. We and others have previously shown that the recruitment of neutrophils into ischemic hemispheres depends on the IL-17A production by γδ T cells, which in turn induces C-X-C motif chemokine ligand 1 production by astrocytes.[2] After depletion of CD11c$^+$ cells, we observed a significant decrease of IL-17 levels in brain-infiltrating γδ T cells on days 1 and 3, which was paralleled by increased IFN-γ (interferon-γ) levels in γδ T cells on day 3 (Figure 4C; Figure IIIB in the online-only Data Supplement), whereas cytokine levels in infiltrating αβ T cells were not altered. Consistent with the reduction in IL-17, we detected significantly decreased *Cxcl1* levels in DTX-treated *Cd11c.DOG* mice (Figure 4D).

Infiltrating IL-17$^+$ γδ T Cells Express the IL-23R

A potential regulator of IL-17 production in T cells is IL-23.[16] To address the role of IL-23 in stroke, we analyzed a knock-in reporter mouse, in which an IRES GFP (internal ribosomal entry site green fluorescent protein) cassette was introduced in the endogenous IL-23R (IL-23 receptor) gene locus.[17] In heterozygous mice, IL-23R–expressing cells can be visualized by their GFP expression (*Il23r.gfp.KI*). When bred as homozygotes (*Il23r*$^{-/-}$), the deletion of the IL-23R abrogates their responsiveness to IL-23. In our model, 3 days after tMCAO, we observed that ≈80% of the brain-infiltrating IL-17A$^+$ γδ T cells coexpressed the IL-23R (Figure 5A).

Il23r$^{-/-}$ Mice Have a Defective IL-17 Response in γδ T Cells and Are Protected From Ischemic Stroke

Neutrophil infiltration, frequency of IL-17–producing γδ T cells, *Cxcl1* expression, and infarct sizes were significantly decreased in *Il23r*$^{-/-}$ mice after tMCAO (Figure 5A through 5C). In contrast, infiltration of macrophages, DCs and T-cell subpopulations, expansion of microglia, IFN-γ production in αβ T cells, and mortality rate were not affected by the IL-23R deficiency (Figure 5A and 5B; Figure IVB and IVC in the online-only Data Supplement). To underline the importance of the IL-23R on T cells, we transferred unfractionated T cells from *Il23r*$^{+/+}$ or *Il23r*$^{-/-}$ mice to T cell–deficient mice (*Rag1*$^{-/-}$ mice). Three days after stroke, neutrophil infiltration into ischemic hemispheres was significantly reduced after transfer of *Il23r*$^{-/-}$ T cells compared with *Il23r*$^{+/+}$ T cells (Figure 5D).

IL-23 Is Rapidly Induced in Brain-Infiltrating cDC2 Cells

To test whether cDC2s released IL-23, we measured levels of *Il23p19* and *Tnf* transcripts in cDC2s, macrophages, and microglia, sorted from ischemic hemispheres and in splenic cDC2s by quantitative real-time polymerase chain reaction (gating strategy in Figure I in the online-only Data Supplement). After normalization to levels in microglia, we found that *Il23p19* transcripts were significantly upregulated in cDC2s in comparison with infiltrating macrophages and resident microglia at 24 hours, whereas *Tnf* levels were reduced in cDC2s (Figure 6A). Comparing the *Il23p19* levels from brain-derived cDC2s with splenic cDC2s, we found a 70-fold increase in cDC2s derived from ischemic hemispheres (Figure 6B). In congruence, we found reduced expression levels of *Il23p19* transcripts in ischemic hemispheres of *CD11c.DOG* mice after depletion of CD11c$^+$ cells (Figure 6C). To confirm that infiltrating cDC2s and not microglia are the main source of IL-23, we generated bone marrow chimeric mice. Wild-type animals reconstituted with *Il23p19*$^{-/-}$ bone marrow showed a significantly reduced neutrophil infiltration into ischemic hemispheres (Figure 6D), underscoring the essential expression of IL-23 in peripheral immune cells.

Discussion

In this study, we identify IRF4$^+$/CD172a$^+$ DCs (cDC2s) as the predominant DC subset in the ischemic brain, and major source of IL-23, which in turn promotes IL-17 production in γδ T cells and the subsequent recruitment of neutrophils to the ischemic hemisphere. Thus, cDC2s orchestrate the early phase of poststroke inflammation. Disruption of the IL-23–IL-17 pathway, either by depletion of CD11c$^+$ cells or by genetic ablation of the IL-23R, resulted in a significantly reduced frequency of IL-17–producing γδ T cells in ischemic brains and improved the neurological outcome after stroke. Moreover, our analysis of human pathological tissue revealed that DCs are present in human stroke tissue.

DCs had already been described as part of the infiltrating immune cells in stroke, but their phenotype and immunologic function remained elusive. These CD11c$^+$ cells were primarily described as CD45^{high}/CD11b$^+$/CD8α$^-$ and located both in direct proximity of the stroke but also in remote areas.[8] According to the new DC nomenclature proposed by Guilliams et al,[11] we can now classify the infiltrating DCs in stroke based on their phenotype, function, and lineage-specific transcription markers. Applying this classification, we found that CD172a$^+$ cDC2s were the predominant subpopulation already at 24 hours after stroke, outnumbering monocyte-derived DCs, plasmacytoid DCs, and cDC1s. We further confirmed the identity of the cDC2s based on high expression levels of the developmental transcription factors *Irf4* and *Zbtb46* in combination with low levels of *Irf8* and *Batf3*.[18,19]

The observation of an early protection from ischemic stroke after depletion of CD11c$^+$ cells is in line with a central role of these rapidly infiltrating cDC2s in the initiation of sterile inflammation. We found that the cDC2s served as an essential early source of IL-23. The subsequent IL-23–dependent immune response is similar to the initiation of inflammation during infection.[20] In short, IL-23 drives and sustains IL-17 production in γδ T cells, which in turn triggers mechanisms for neutrophil recruitment. γδ T cells are lymphocytes, which can be rapidly activated in a T cell receptor-independent manner. Interestingly, others and we have shown that a specific subpopulation of γδ T cells, so called natural IL-17–producing γδ T cells, is responsible for the rapid IL-17 production in models of stroke, infection, and autoimmunity.[20,21] These IL-17$^+$ γδ T cells also carry the IL-23R and, therefore, can be activated by IL-23.[20] In other organ systems, *Irf4*-expressing cDC2 cells are also specialized in driving IL-17 responses. These cells are known to induce IL-17 responses in the lung and the intestine.[22] However, our finding that the depletion of CD11c$^+$ cells improved survival, whereas the genetic ablation of the IL-23R did not reveal significant effects on mortality, indicates that CD11c$^+$ cells exert their detrimental function not only through IL-23 but also other mechanisms.

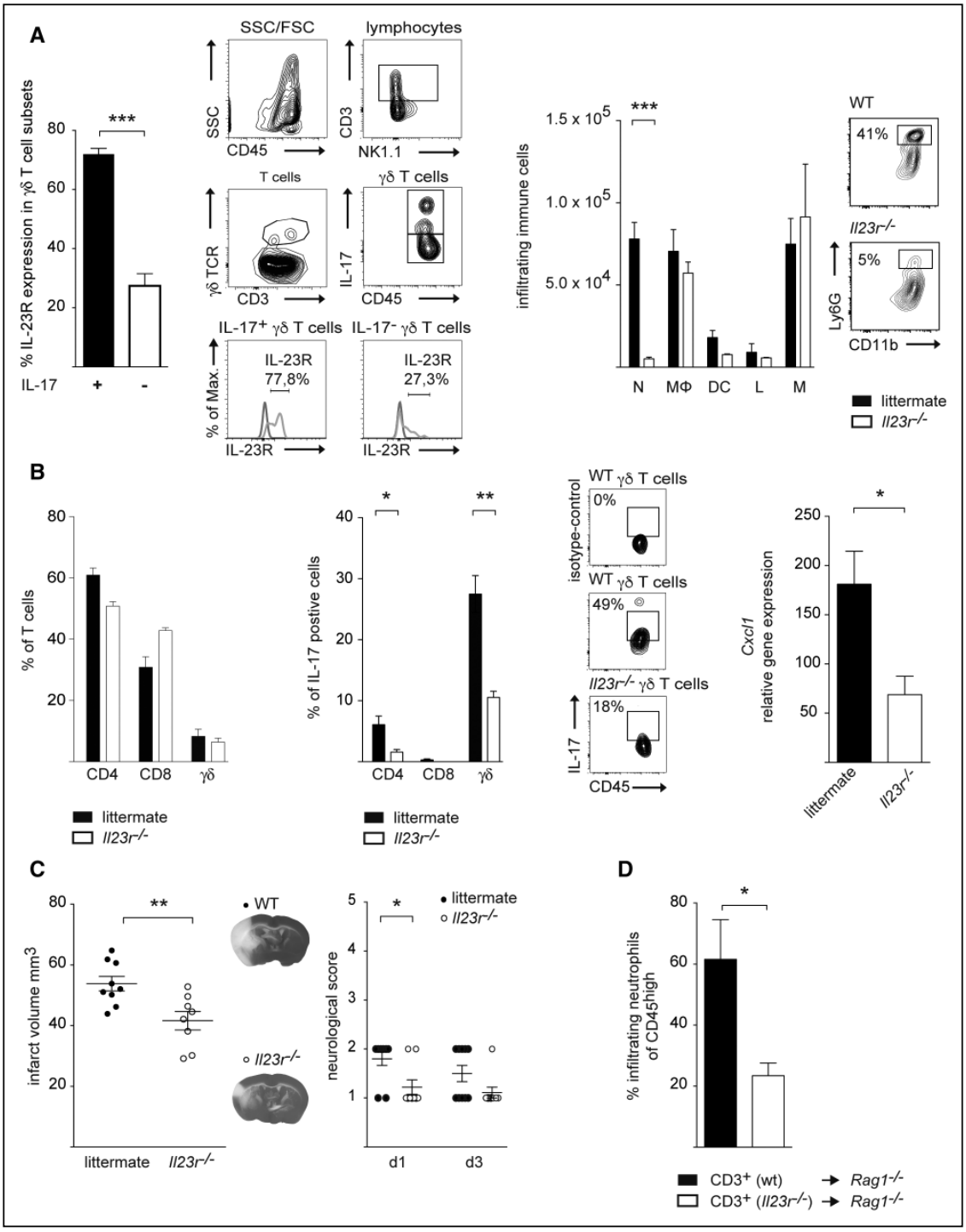

Figure 5. Disruption of IL-23 (interleukin-23) signaling is protective in ischemic stroke. **A**, Flow cytometric analysis of the IL-23R (IL-23 receptor) expression in IL-17⁺ and IL-17⁻ γδ T-cell subsets isolated from ischemic hemispheres of *Il23r.gfp.KI* mice, and flow cytometric analysis of absolute numbers of neutrophils (N), macrophages (MΦ), dendritic cells (DC), lymphocytes (L), and microglia (M) isolated from ischemic hemispheres of littermate controls and *Il23r*$^{-/-}$ mice 3 days after transient middle cerebral artery occlusion (tMCAO). Lymphocytes from *Il23r.gfp.KI* mice were stained for CD45, CD3, NK1.1, and γδ T cell receptor followed by an intracellular staining for IL-17. Histograms show expression of IL-23R in IL-17–positive and negative γδ T cells of 1 representative experiment at day 3. Central nervous system-infiltrating cells were stained for CD45, Ly6G, CD11b, CD11b, MHCII, B220, CD3, NK1.1, CD4, CD8, and γδ T cell receptor. **B**, Frequency of CD4⁺, CD8⁺, and γδ T cells, flow cytometric analysis of IL-17 produced by CD4⁺, CD8⁺, and γδ T cells isolated from ischemic hemispheres of littermate controls and *Il23r*$^{-/-}$ mice 3 days after tMCAO, and relative gene expression of *Cxcl1* in ischemic hemispheres of littermate controls and *Il23r*$^{-/-}$ mice 24 hours after tMCAO. **C**, Triphenyltetrazolium chloride (TTC) staining for evaluation of infarct volume at day 3 (**left**), and neurological scores at days 1 and 3 (**right**) of littermate controls and *Il23r*$^{-/-}$ mice after middle cerebral artery occlusion (MCAO). Data are presented as mean±SD of 8 littermate controls and 8 *Il23r*$^{-/-}$ animals. **D**, Percentage of neutrophils in central nervous system-infiltrating cells of *Rag1*$^{-/-}$ mice reconstituted 1 hour before stroke induction with 1×10^7 CD3⁺ cells isolated from wild-type (WT) or *Il23r*$^{-/-}$ mice. **A**, **B**, and **D**, Data show the mean±SD of 4 to 8 animals per group, analyzed in 3 to 4 independent experiments. Statistical significances were analyzed by Student *t* test (**A**–**D**) and Mann–Whitney *U* test for neurological scores (**C**). **P*<0.05, ***P*<0.01, ****P*<0.001. FSC indicates forward scatter; and SSC, side scatter.

Previously, infiltrating macrophages were believed to be the main source of IL-23 in stroke.[4] This apparent discrepancy can be explained by the gating strategy applied for the fluorescence-activated cell sorting of macrophages and microglia in previous studies.[4] Macrophages were identified by gating on CD45^{high}/CD11b^{+} cells—a strategy that included IL-23–producing cDC2s. Furthermore, based on our chimera experiments, we can now attribute the IL-23/IL-17–mediated effects to radiation-sensitive peripheral immune cells.

Throughout the study, we focused on the role of cDC2s because of their rapid infiltration kinetics and significantly increased abundance in comparison with all other DC subsets. Nevertheless, we also observed cDC1s with delayed infiltration kinetics. Even though the immunologic function of the cDC1 cells is unclear, a regulatory role for XCR1^{+}/CD172a^{-} cDC1s can be speculated. Recent experimental data suggest that CD103^{+} DCs from the small intestine can shape the IL-17 response in T cells. In a model of experimental stroke, alterations of the intestinal flora led to a decrease in IL-17–positive γδ T cells in the ischemic brain. These changes could be traced back to an increase of CD103^{+} DCs in the gut. The CD103^{+} are likely to belong to the cDC1 subset, which we found in ischemic hemisphere at later stages.[23]

In conclusion, our study provides further insights into the inflammatory cytokine and immune cell networks of post-stroke inflammation and suggests that rapidly infiltrating IRF4^{+}/CD172a^{+} cDC2s are an essential source of IL-23. These findings indicate that cDC2s are critical for the initiation of the detrimental IL-17–driven innate immune response in stroke.

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Disclosures

None.

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Supplemental Material

GELDERBLOM *et al.* Running title: DCs in stroke

IL-23 producing conventional dendritic cells control the detrimental IL-17 response in stroke.

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Supplemental methods

In vivo stroke model

We conducted transient middle cerebral artery occlusion (tMCAO) for 45 min using the intraluminal filament method (6-0 nylon) as described before[1]. Heterozygous male *Cd11c.DOG* mice[2] were kindly provided by Natalio Garbi, Germany, and heterozygous and homozygous male IL-23R reporter mice by Thomas Korn[3]. *Cd11c-EYFP* mice were obtained from The Jackson Laboratory. Mice for all animal experiments were randomized and coded by an independent researcher, so experiments were carried out blindly. Transgenic mice were back-crossed at least 10 generations to the C57BL/6 background. Age-matched male wildtype (WT) littermates served as controls. To reduce the variability of our outcome parameters (neurological outcome, inflammatory response) caused by sex-differences and to thereby decrease group sizes, only male mice were used throughout the study. Recent studies have demonstrated profound effects of sex-differences on infarct sizes and the inflammatory response[4]. To validate our studies in females, further studies are required. We monitored mice for heart rate, respiratory rate, oxygen saturation, and rectal body temperature. To exclude an insufficient occlusion of the middle cerebral artery in our stroke model in littermate controls, *Il23r*$^{-/-}$ and *Cd11c.DOG* mice we performed laser doppler and time of flight angiography, which showed no differences between genotypes **(Figure IVA in the online-only Data Supplement)**. Depletion of DCs was achieved by i.p. injection of diphtheria toxin 8ng/gBW starting 1 day before tMCAO or sham surgery, which was continued by daily injection of 8ng/gBW. Exclusion criteria were defined in a dropout score, which included weight loss, general condition, spontaneous behavior, and impairment of wound healing. Numbers of included / omitted mice are shown in Table II (**in the online-only Data Supplement)**. We anesthetized all mice (20 to 25 g, 12 weeks; TVH, University Medical Center Hamburg-Eppendorf) using isoflurane 1% to 2% v/v oxygen and we injected buprenorphine 0.03 mg/kgBW intraperitoneally (i.p.) every 12 hours for 24 hours as analgesia. After stroke induction, we repeatedly scored every mouse on a scale from 0–5 immediately after reawakening and every day until sacrifice. Bederson Score: 0 no deficit, 1 preferential turning, 2 circling, 3 longitudinal rolling, 4 no movement, 5 death. Mice were sacrificed one, three, or seven days after reperfusion using isoflurane and decapitation. Only mice with a Bederson score greater than or equal to one after reawakening, and an sufficient occlusion of the middle cerebral artery during MCAO as measured by laser doppler were included for stroke size analysis. Sample size calculation was performed (stroke size from pilot experiments, significance level 0.05, power 90%) and resulted in 8 animals per group to see a difference of 23% in stroke size.

Analysis of infarct size by TTC staining

We analyzed infarct size by harvesting brains and cutting them into 1 mm slices (Braintree Scientific, 1mm) followed by vital staining using 2% (w/v) 2,3,5-triphenyl-2-hydroxy-tetrazolium chloride (TTC) in phosphate buffer. We determined infarct volumes in a blinded fashion using NIH ImageJ software.

Stroke assessment by magnetic resonance imaging

Magnetic resonance imaging (MRI) was performed on a dedicated 7T MR small animal imaging system (ClinScan, Bruker). The image protocol comprised T2-

weighted imaging, diffusion-weighted imaging (DWI), and 3D time-of-flight (TOF) angiography.

Antibodies and flow cytometry

Flow cytometry for the analysis of cell types was performed as previously described 3. T cells were stimulated with phorbol 12-myristate 13-acetate (100 ng/mL; Sigma-Aldrich) and ionomycin (1 µg/mL; Sigma-Aldrich) in the presence of brefeldin A (3 µg/mL; eBioscience) for 4 hours. Mouse antibodies were as follows: from eBioscience B220 (RA3-6B2), CD3 (145-2C11), CD4 (GK1.5), CD8a (53-6.7), CD11b (M170), CD11c (N418), TCR-γδ (GL-3), NK1.1 (PK136), CD45 (30-F11), Ly6G (1A8), MHCII (M5/114.15.2), IL-17A (17B7), IFN-γ (XMG1.2), TNF-α (MP6-XT22); from Biolegend F4/80 (BM8), CD103 (2E7), Ly6C (HK1.4), XCR1 (ZET); from BD CD172a (P84) from R&D Systems CCR2 (475301). For flow cytometry analysis animals were euthanized and perfused with phosphate-buffered saline. Only ipsilesional hemispheres were dissected, digested for 30 min at 37°C (1 mg/ml collagenase (Roche), 0.1 mg/ml DNAse I (Roche) in DMEM), and pressed through a cell strainer. Cells were incubated with standard erythrocyte lysis buffer on ice and separated from myelin and debris by Percoll gradient (GE Healthcare) centrifugation. For absolute quantification, TrueCount tubes (Becton Dickinson) containing fluorescence beads were used according to the manufacturer's protocol and 10% of the sample volume was counted. Data were acquired with a Fortessa FACS system (BD Biosciences) and analyzed with FlowJo (TreeStar). Doublets were excluded with FSC-A and FSC-H linearity.

Cell Sorting and RNA isolation

Immune cells were sorted using a BD FACS Aria IIIu and collected in RPMI with 25% fetal calf serum (FCS). We isolated total RNA from cells using QIA-Shredder spin columns and the RNeasy Micro Kit (QIAGEN) and transcribed complementary DNA using Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Fermentas).

RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction

We isolated RNA from brain tissue by killing the mice with isoflurane at indicated time points (24 hours) after stroke induction. Hemispheres were separated and homogenized in TRIzol Reagent (1ml per 100 mg tissue), chlorophorm was added, samples were centrifuged at 12000 g for 15 min at 4 °C and the upper aqueous phase was collected. RNA was precipitated by addition of isopropyl alcohol, washed and dissolved in TE-Buffer. We isolated total RNA from cells using QIA-Shredder spin columns and the RNeasy Micro Kit (QIAGEN) and transcribed complementary DNA using Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Fermentas). We obtained real-time PCR primers from Applied Biosystems (Carlsbad, CA): *Batf3*: Mm01318274_m1; *Cxcl1* Mm00433859_m1; *Il23*: Mm00518984_m1; *Irf4*: Mm00516431_m1; *Irf8*: Mm00492567_m1; *Sdha*: Mm01352366_m1; *Tnf*: Mm00443258_m1; *Zbtb46*: Mm00511327_m1. We purchased probe mixtures from Fermentas (Waltham, MA). The relative gene expression was calculated using ΔΔCt method, and the samples were normalized to control population and to the expression of *Sdha.* Samples were randomized and coded by an independent researcher, so experiments were carried out blindly.

Immunohistochemistry

For histological analysis of mouse brains, animals were perfused with 4% buffered formalin. Brains were embedded in paraffin and 3 µm thick brain sections were

stained following standard immunohistochemistry procedures using the Ventana Benchmark XT machine (Ventana) with antibodies against GFAP (1:200, DAKO) and Iba-1 (1:200, Wako) followed by detection with anti-rabbit histofine Simple Stain MAX PO Universal immuno-peroxidase polymer (Iba-1, Nichirei Biosciences, Tokyo, Japan) or Mouse Stain Kit (GFAP, Nichirei). Visualization of secondary antibodies was performed using the "Ultra View Universal DAB Detection Kit" from Ventana. For immunostaining of neutrophils, Ly-6G clone 1A8 (1:1000, Biolegend) was used overnight at 4°C following antigen retrieval in 10 mM citrate buffer (pH 6.0). Counterstaining was performed with Mayer´s haematoxylin solution.
For the analysis of autoptic human brain tissue, cases were selected from the files of the Institute of Neuropathology at the University Medical Center Hamburg-Eppendorf. Brain specimens had been fixed in 4% buffered formalin for at least 3 weeks before paraffin-embedding. Brain sections (3 µm thick) were stained according to standard immunohistochemistry procedures with antibodies against CD11c (Abcam, ab52632, rabbit monoclonal, 1:500) and CD11b (Abcam, ab52478, rabbit monoclonal, 1:500). Samples were randomized and coded by an independent researcher, so experiments were carried out blindly.

Immunofluorescence
For immunofluorescence analysis, animals were perfused with 4% buffered formalin. The brain was removed, fixed overnight with the same fixative, and immersed in 30 % sucrose in PB and frozen in isopentane at -40 ºC. Cryostat brain sections (14-µm-thick) were fixed in ethanol, blocked with normal serum, and incubated overnight at 4º C with a rabbit polyclonal antibody against pan-laminin (1:100, DAKO), followed by the secondary antibodies Alexa Fluor 546 (Molecular Probes). The fluorescence of EYFP-CD11c+ cells was observed at 514 nm without any enhancement. Cell nuclei were stained with Hoechst or To-Pro3 (Invitrogen) (shown in blue). Images were obtained by confocal microscopy (SP5 or TCS SPE-II, Leica Microsystems) with the LAS software (Leica) and where were not further processed excepting for enhancing global signal intensity in the entire images for image presentation purposes. Samples were randomized and coded by an independent researcher, so experiments were carried out blindly.

Bone marrow chimeras
For the generation of bone marrow chimeras in WT controls or *Il23p19*$^{-/-}$ mice with either *Il23p19*$^{-/-}$ or WT bone marrow respectively, 8-week-old male recipients were irradiated by whole-body irradiation (9 Gy; 1 Gy min−1) using a cesium-137 gamma irradiator (BIOBEAM 2000, Leipzig, Germany) as described before [5]. After 24 h we reconstituted them with bone marrow cells derived from tibiae and femurs from *Il23p19*$^{-/-}$ or WT mice. Every recipient received 1×10^7 bone marrow cells intravenously. We induced tMCAO in recipient mice six weeks after transplantation.

Stroke Online Supplement

Table I. Checklist of Methodological and Reporting Aspects for Articles Submitted to *Stroke* Involving Preclinical Experimentation

Methodological and Reporting Aspects	Description of Procedures
Experimental groups and study timeline	☒ The experimental group(s) have been clearly defined in the article, including number of animals in each experimental arm of the study. ☒ An account of the control group is provided, and number of animals in the control group has been reported. If no controls were used, the rationale has been stated. ☒ An overall study timeline is provided.
Inclusion and exclusion criteria	☒ A priori inclusion and exclusion criteria for tested animals were defined and have been reported in the article.
Randomization	☒ Animals were randomly assigned to the experimental groups. If the work being submitted does not contain multiple experimental groups, or if random assignment was not used, adequate explanations have been provided. ☒ Type and methods of randomization have been described. ☒ Methods used for allocation concealment have been reported.
Blinding	☒ Blinding procedures have been described with regard to masking of group/treatment assignment from the experimenter. The rationale for nonblinding of the experimenter has been provided, if such was not feasible. ☒ Blinding procedures have been described with regard to masking of group assignment during outcome assessment.
Sample size and power calculations	☒ Formal sample size and power calculations were conducted based on a priori determined outcome(s) and treatment effect, and the data have been reported. A formal size assessment was not conducted and a rationale has been provided.
Data reporting and statistical methods	☒ Number of animals in each group: randomized, tested, lost to follow-up, or died have been reported. If the experimentation involves repeated measurements, the number of animals assessed at each time point is provided, for all experimental groups. ☒ Baseline data on assessed outcome(s) for all experimental groups have been reported. ☒ Details on important adverse events and death of animals during the course of experimentation have been provided, for all experimental arms. ☒ Statistical methods used have been reported. ☒ Numeric data on outcomes have been provided in text, or in a tabular format with the main article or as supplementary tables, in addition to the figures.
Experimental details, ethics, and funding statements	☒ Details on experimentation including stroke model, formulation and dosage of therapeutic agent, site and route of administration, use of anesthesia and analgesia, temperature control during experimentation, and postprocedural monitoring have been described. ☒ Different sex animals have been used. If not, the reason/justification is provided. ☒ Statements on approval by ethics boards and ethical conduct of studies have been provided. ☒ Statements on funding and conflicts of interests have been provided.

Table II

	Cd11c.DOG + DTX *Incl. / omitted*	*littermate for Cd11c.DOG + DTX* *Incl. / omitted*	*Il23r.gfp.KI and Il23r$^{-/-}$* *Incl. / omitted*	*littermate for Il23r$^{-/-}$* *Incl. / omitted*	C57Bl/6 *Incl. / omitted*	*Cd11c*-EYFP *Incl. / omitted*	*Il23p19$^{-/-}$* *Incl. / omitted*	*Rag1$^{-/-}$* *Incl. / omitted*
Infarct size after tMCAO:								
D3			**8 / 1**	**8 / 4**				
D1	**16 / 1**	**12 / 8**						
Flow cytometry Brain / Spleen / Blood:								
Sham	**3 / 0**	**3 / 0**			**4 / 0**			
12 hours					**6 / 2**			
D1	**15 / 3**	**16 / 4**			**9 /4**			
D3	**12 / 2**	**12/ 5**	**15/3**	**16 / 7**	**4 / 2**			
D7					**4 / 3**			
Sorting for transcriptome and cytokine analysis of DC subtypes, macrophages and microglia:								
12 hours					**8 / 5**			
24 hours					**9 / 7**			
spleen					**7 / 4**			
Immunohistochemistry:								
	3 / 1	**3 / 2**				**5 / 2**		
Bone marrow chimeras and FACS analysis:								
Donor					**8 / 0**		**8 / 0**	
Acceptor					**12 / 4**		**12 / 4**	
T cell transfer and FACS analysis:								
Donor			**4 / 0**	**4 / 0**				
Acceptor								**6 / 2**
Whole brain RNA analysis:								
Sham	**5 / 0**	**5 / 0**	**5 / 0**	**5 / 0**				
D1	**6 / 2**	**6 / 3**	**8 / 2**	**8 / 3**				

Table II: Experimental Groups.

Figure I

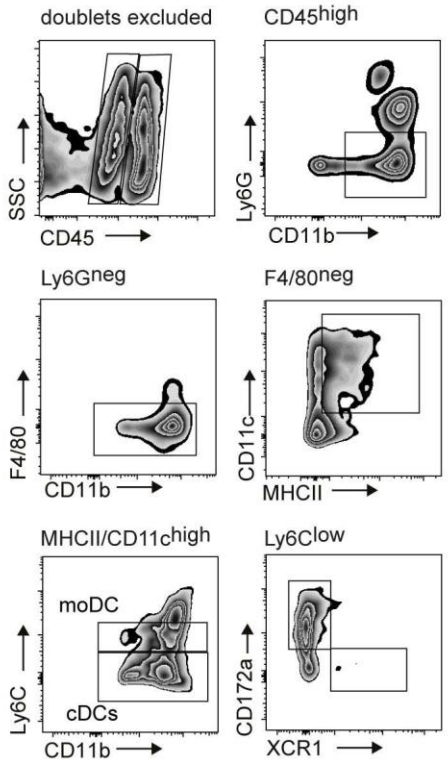


Figure I: Sorting strategy to identify CD172a⁺/XCR1⁻ cDC2 cells.
Data were obtained by flow cytometric analysis of CNS-infiltrating cells purified from ischemic hemispheres 24 h following MCAO after Percoll density centrifugation. Cells were stained for CD45, CD11b, CD11c, MHCII, CD172a, Ly6c, Ly6g, F4/80, XCR1.

Figure II

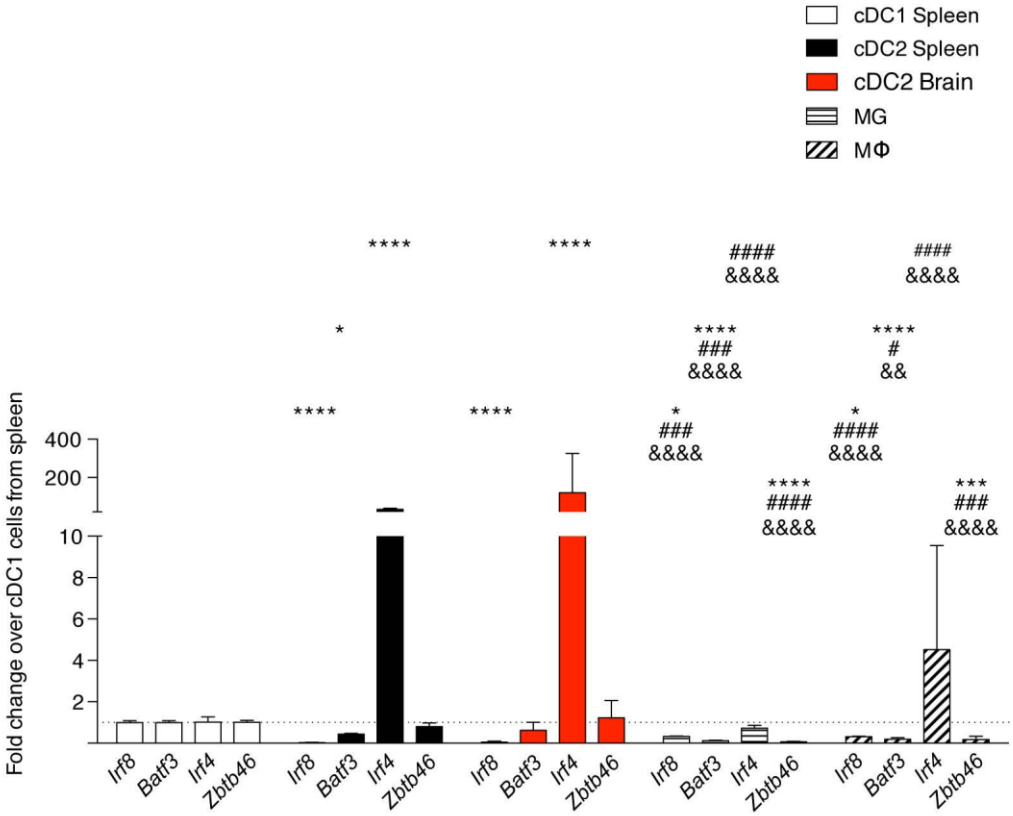
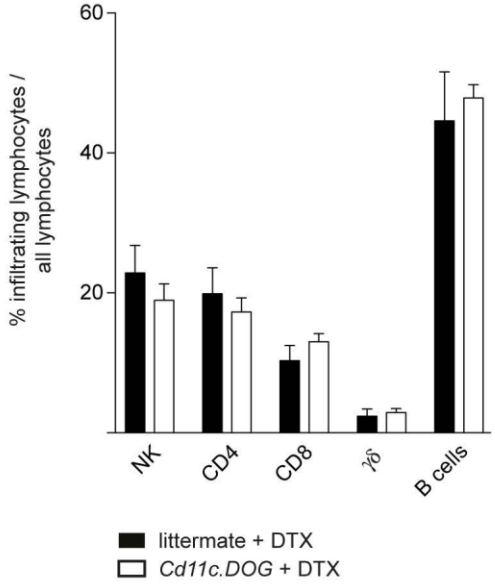


Figure II: Classification of infiltrating dendritic cell subsets by analysis of ontogenetic markers. Relative gene expression of *Irf4*, *Irf8*, *Batf3*, and *Zbtb46* in CNS-infiltrating and splenic CD45^{high}/CD11b^{+}/CD11c^{+}/MHCII^{+}/CD172a^{+}/Ly6c^{-}/Ly6g^{-}/F4/80^{-}/XCR1^{-} cDC2, CD45^{intermed}/CD11b^{+} microglia and CNS-infiltrating CD45^{high}/CD11b^{+}/CD11c^{-}/MHCII^{-}/Ly6g^{-}/F4/80^{+} macrophages purified from ischemic hemispheres and spleens 12 h following tMCAO by FACS sorting. Expression levels were normalized to corresponding levels of splenic cDC1s. The graphs show mean ± SD of 5-8 animals per group, in 5-7 independent experiments for each time point. Statistical significances analyzed by One way ANOVA with Bonferroni posthoc test.
*P<0.05, **P<0.01, ***P<0.001, and **** P<0.0001. * vs. cDC1 spleen; # vs. cDC2 spleen; & vs. cDC2 brain.

Figure III

A



B

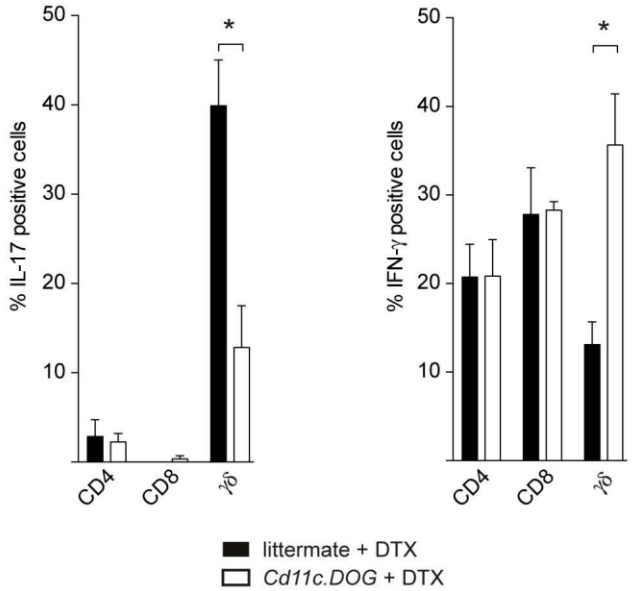


Figure III: Depletion of CD11c⁺ cells alters the production of IL-17 by γδ T cells.
(A) Frequency of CD4⁺, CD8⁺, γδ T cells, NK cells, and B cells in ischemic hemispheres of DTX-treated littermate controls and *Cd11c.DOG* mice 3 days after MCAO. **(B)** Flow cytometric analysis of IL-17 and IFN-γ produced by CD4⁺, CD8⁺ and γδ T cells isolated from ischemic hemispheres at day 3 after stroke induction. **(A, B)** Cell counts were determined by flow cytometric analysis. Daily DTX administration of 8ng/g body weight DTX was started 1 day prior to MCAO. Data show the mean ± SD of 9-12 animals per group, analyzed in 3-4 independent experiments. Statistical significances were analyzed by Student *t*-test in all cases. *$P<0.05$.

Figure IV

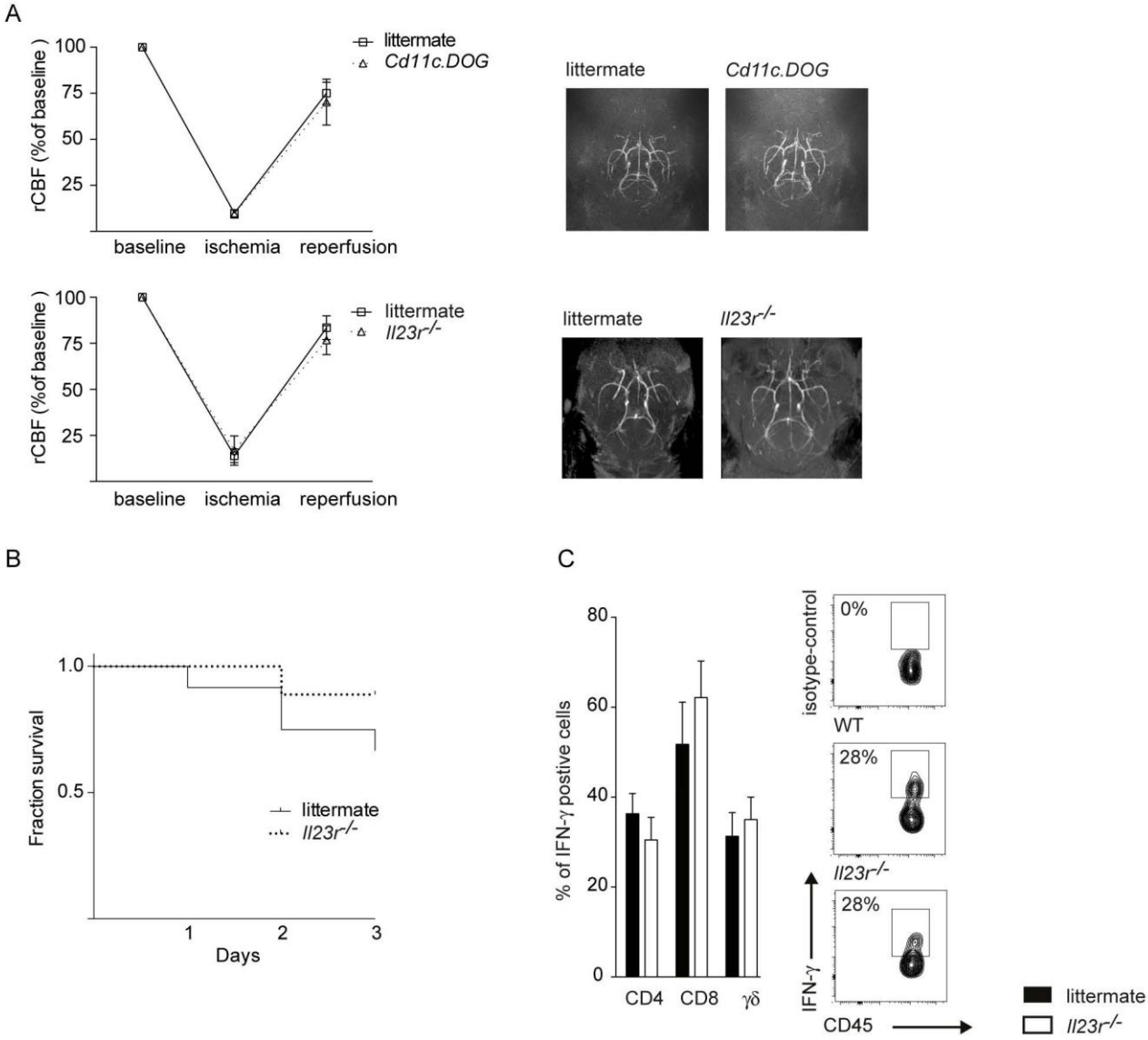


Figure IV: Time of flight angiography, laser Doppler measurements, and analysis of IFN-γ produced by CD4⁺, CD8⁺ and γδ T cells in *Il23*$^{-/-}$ mice and littermate controls.
(A) In all mice subjected to MCAO, regional cerebral blood flow (rCBF) was measured with Laser Doppler in littermate controls, *Cd11c.DOG* mice, and *Il23r*$^{-/-}$ mice. Data show the mean ± SD of 6-8 animals per group, analyzed in 6-8 independent experiments. The Circle of Willis was visualized by time of flight angiography in littermate controls, *Cd11c.DOG* mice, and *Il23r*$^{-/-}$ mice.
(B) Survival was recorded over a time course of 3 days in *Il23r*$^{-/-}$ mice and littermate controls.
(C) Flow cytometric analysis of IFN-γ produced by CD4⁺, CD8⁺ and γδ T cells isolated from ischemic hemispheres of littermate controls and *Il23r*$^{-/-}$ mice 3 days after MCAO. Flow cytometry panels show intracellular stainings for IFN-γ of gated CD4⁺, CD8⁺, and γδ T cells of 1 representative experiment. Data show the mean ± SD of 4-8 animals per group, analyzed in 3-4 independent experiments. **(A, C)** Statistical significances were analyzed by Student *t*-test, and **(B)** $\chi 2$ test (survival rate). *P*-values ≤ 0.05 were considered significant.

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Dendritic Cells and Microglia Have Non-redundant Functions in the Inflamed Brain with Protective Effects of Type 1 cDCs

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SUMMARY

Brain CD11c$^+$ cells share features with microglia and dendritic cells (DCs). Sterile inflammation increases brain CD11c$^+$ cells, but their phenotype, origin, and functions remain largely unknown. We report that, after cerebral ischemia, microglia attract DCs to the inflamed brain, and astroglia produce Flt3 ligand, supporting development and expansion of CD11c$^+$ cells. CD11c$^+$ cells in the inflamed brain are a complex population derived from proliferating microglia and infiltrating DCs, including a major subset of OX40L$^+$ conventional cDC2, and also cDC1, plasmacytoid, and monocyte-derived DCs. Despite sharing certain morphological features and markers, CD11c$^+$ microglia and DCs display differential expression of pattern recognition receptors and chemokine receptors. DCs excel CD11c$^-$ and CD11c$^+$ microglia in the capacity to present antigen through MHCI and MHCII. Of note, cDC1s protect from brain injury after ischemia. We thus reveal aspects of the dynamics and functions of brain DCs in the regulation of inflammation and immunity.

INTRODUCTION

Dendritic cells (DCs) are a heterogeneous population of cells in phenotype and origin, with tissue-specific features (Schlitzer et al., 2015). On the basis of ontogeny and function, DCs are classified as conventional DCs (cDCs) and plasmacytoid DCs (pDCs) under steady state. A further classification of cDCs is based on the expression of mutually exclusive markers: either XCR1 and DNGR-1 (*Clec9a*) (cDC1 lineage) or CD11b^{hi} and SIRPα (cDC2 lineage) (Guilliams et al., 2014). Fate-mapping studies demonstrated the existence of a DC hematopoietic lineage by identifying a common DNGR-1$^+$ DC precursor unable to generate monocytes and able to generate cDC1s and cDC2s to a different extent (Schraml et al., 2013). Inflammatory conditions induce the generation of inflammatory DCs (iDCs) that may derive from monocytes (Domínguez and Ardavín, 2010), but their origin is still unclear. DCs capture and present antigens, and their role is critical not only for mounting adaptive immune responses against invading pathogens but also for establishing tolerance to self-antigens under steady state. DCs traffic between tissues and lymphoid organs through the lymphatic system or they access different compartments through the blood vessels. Despite specific anatomic features of the central nervous system (CNS) (Ransohoff and Engelhardt, 2012), DCs are found surrounding the brain parenchyma, in brain regions proximal to the cerebrospinal fluid (CSF), and zones with an incomplete blood-brain barrier (i.e., at the frontline of interaction between brain and periphery). These regions comprise the leptomeninges, ventricles, choroid plexus, circumventricular organs, and rostral migratory pathway (Anandasabapathy et al., 2011; Bulloch et al., 2008; D'Agostino et al., 2012; Mohammad et al., 2014). Notably, cross-presenting cDC1s are found within meningeal/choroid plexus CD11c$^+$ cells in steady state (Anandasabapathy et al., 2011; Quintana et al., 2015). Besides these specific

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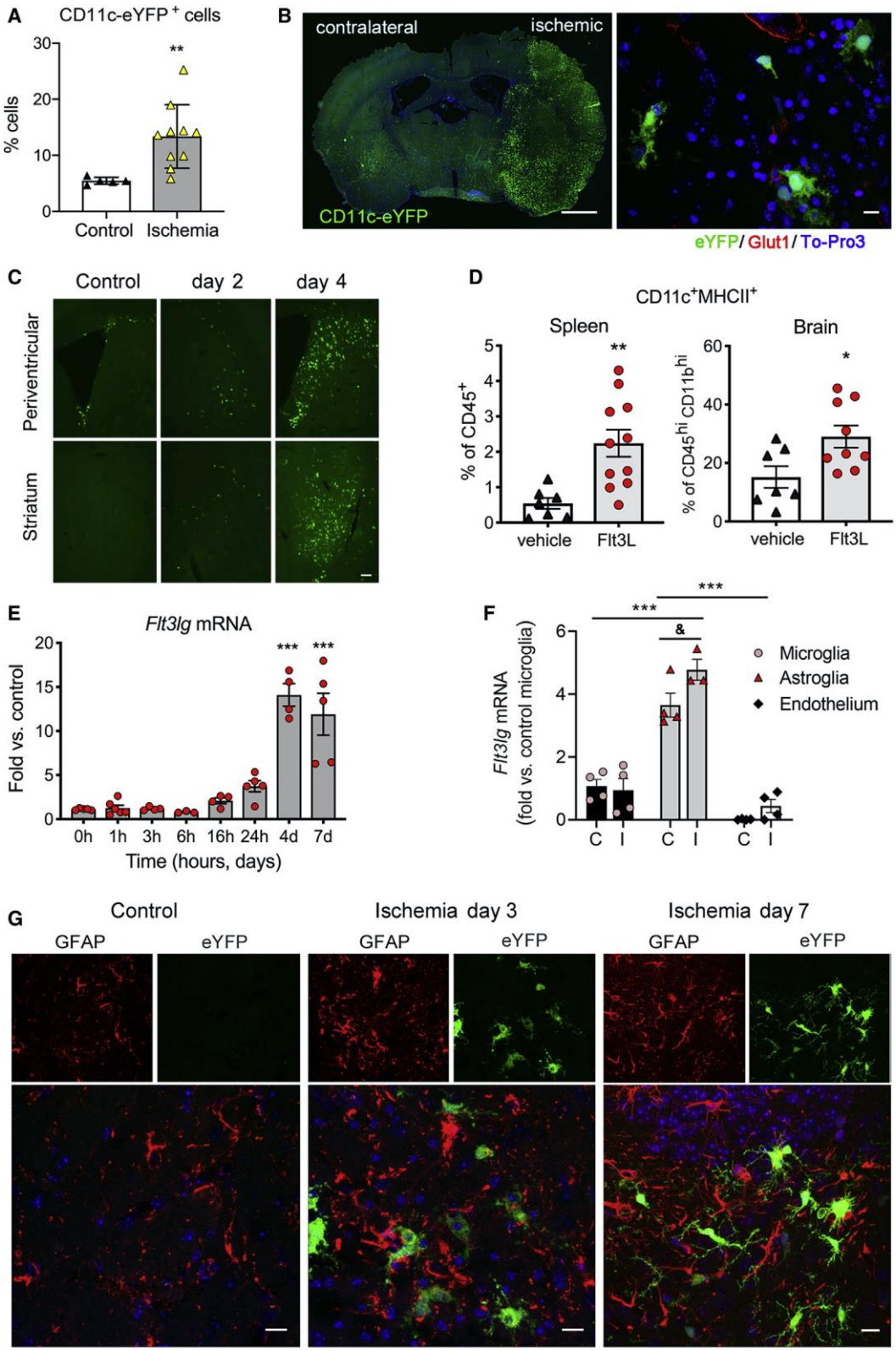

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locations, CD11c$^+$ cells are rarely found in adult brain parenchyma under physiological conditions. However, their presence increases in the aged brain (Kaunzner et al., 2012) and under neuroinflammatory and neuropathological conditions, including infection, cancer, autoimmunity, seizures, neurodegenerative diseases, and stroke (Clarkson et al., 2012; D'Agostino et al., 2012; Ludewig et al., 2016).

Stroke causes acute sterile inflammation due to necrotic neural cell death triggered by sudden interruption of blood supply to certain brain regions (Chamorro et al., 2012, 2016; Dirnagl et al., 1999; Iadecola and Alexander, 2001; Iadecola and Anrather, 2011). CD11c$^+$ cell number increases in brain parenchyma in rodent models of ischemic stroke (Felger et al., 2010; Kostulas et al., 2002; Prodinger et al., 2011). Although some studies suggested that these cells may be microglia derived (Dando et al., 2016; Reichmann et al., 2002), a previous study demonstrated that about 50% of the CD11c$^+$ cells found in the ischemic brain infiltrated from the periphery (Felger et al., 2010). However, knowledge about the phenotype and function of resident and infiltrating CD11c$^+$ cells in the inflamed brain is still very limited. The objective of this study was to gain insight into the features and functions of brain resident and infiltrating CD11c$^+$ cells in the inflamed brain using a stroke mouse model of transient middle cerebral artery occlusion (tMCAo), transcriptomic analysis, cell phenotyping, immune assays, and generation of parabiotic (PA) mice.

RESULTS

Ischemia Increases CD11c$^+$ Cells and Flt3L Expression in Brain

Cerebral ischemia increased CD11c$^+$ cell number in the injured brain of CD11c-eYFP transgenic mice (Figure 1A), particularly at day 4 (Figures 1B and 1C), concurring with previous results (Felger et al., 2010). Fms-related tyrosine kinase 3 ligand (Flt3L) is an important growth factor for DC development and mobilization (Anandasabapathy et al., 2011; Coates et al., 2003; Guermonprez et al., 2013). Systemic Flt3L treatment increased CD11c$^+$ major histocompatibility complex class II (MHCII)$^+$ cells in spleen and ischemic brain (Figure 1D). The injured brain may provide support to CD11c$^+$ cells, as the brain expression of Flt3L mRNA (*Flt3lg*) increased peaking at day 4 post-ischemia (Figure 1E). To find out which brain cells produced Flt3L, we isolated microglia, astroglia, and endothelial cells from controls and 4 days post-ischemia (Figure S1). Astrocytes displayed the strongest *Flt3lg* mRNA expression, which increased after ischemia (Figure 1F). Furthermore, eYFP$^+$ cells were seen in the proximity of reactive astrocytes surrounding the ischemic core (Figure 1G).

Several studies suggested the existence of CD11c$^+$ microglia (Dando et al., 2016; Kamphuis et al., 2016), but the nature of brain CD11c$^+$ cells remains largely unknown. We checked whether cultured glial cells were able to express CD11c after stimulation. *Itgax* mRNA expression was induced from 8 to 48 h after IL-4 treatment, in agreement with a previous study (Butovsky et al., 2007), but not after lipopolysaccharide (LPS) (Figure S2A). In the ischemic tissue, some, but not all, eYFP$^+$ cells shared with microglia several morphological features, common markers, and proliferative capacity (Otxoa-de-Amezaga et al., 2019) (Figure S2B). However, after ischemia, sorted eYFP$^+$ cells showed lower mRNA expression than microglia of typical microglia genes (e.g., *Sall1, Tmem119*, *P2yr12*) (Figure S2C), suggesting differences between CD11c$^+$ cells and microglia.

Differential Transcriptional Signature of CD11c$^+$ Cells versus Microglia in the Ischemic Brain

We then compared the transcriptional profile of eYFP$^+$ cells and microglia sorted using fluorescence-activated cell sorting (FACS) 4 days post-ischemia (Figure 2A; Figure S3). For comparative purposes, we also obtained reference control microglia of non-ischemic mice. As control DCs, we sorted eYFP$^+$ cells from the spleen because of very small numbers in the brain at steady state. Comparative RNA sequencing (RNA-seq) showed that eYFP$^+$ cells of the ischemic brain display a gene expression profile distinct from microglia, both ischemic and control, and from spleen eYFP$^+$ cells (Figure 2B; Figures S4A and S4B). Enrichment analyses highlighted different biological processes

Figure 1. Ischemia Increases Brain CD11c-eYFP$^+$ Cells and Brain Flt3L

(A) Brain eYFP$^+$ cells increase 4 days post-ischemia in CD11c-eYFP mice. Flow cytometry values are percentages of viable cells. Mann-Whitney test, $^{**}p = 0.001$, n = 5 controls, n = 10 ischemic mice.

(B) Left image: coronal brain section of a CD11c-eYFP mouse (n = 6) showing eYFP$^+$ cells (green) in the ischemic hemisphere (right); scale bar, 100 μm. Right image: higher magnification showing eYFP$^+$ cells in parenchyma; blood vessels (Glut1$^+$, red); nuclei (To-Pro3, blue); scale bar, 10 μm.

(C) In control, eYFP$^+$ cells are seen in ventricular region and rostral migratory pathway. After ischemia, eYFP$^+$ cells increase in the parenchyma. Scale bar, 30 μm.

(D) Mice received recombinant mouse Flt3L (10 μg/mouse) or vehicle subcutaneously (s.c.) for 7 days. Ischemia was induced 3 days after treatment onset, and tissue was studied by flow cytometry 4 days post-ischemia. Flt3L increased the percentage of CD11c$^+$MHCII$^+$ cells in spleen (Mann-Whitney test, $^{**}p = 0.001$, n = 7 vehicle, n = 11 Flt3L) and ischemic brain (Mann-Whitney test, $^{*}p = 0.02$, n = 7 vehicle, n = 9 Flt3L). Values are CD11c$^+$MHCII$^+$ cells expressed as percentages of CD45$^+$ cells or CD45^{hi}CD11b^{hi} cells.

(E) Brain Flt3L mRNA (*Flt3lg*) expression increases after ischemia (h, hours; d, days). One-way ANOVA and Dunnett's multiple comparison test, $^{***}p < 0.001$ versus control, n = 3–6 mice per time point.

(F) *Flt3g* mRNA in microglia, astroglia, and endothelial cells sorted from control and ischemic brains (day 4) (n = 3 or 4 samples per cell type and condition). Values are expressed as fold increase versus control microglia. Astrocytes show the highest *Flt3lg* mRNA expression versus microglia ($^{***}p = 0.0003$) and endothelial cells ($^{***}p < 0.0001$), and microglia show higher expression than endothelial cells (two-way ANOVA, $p < 0.001$, Sidak's multiple-comparisons test, $^{***}p = 0.004$). Ischemia 'I' increases astrocyte *Flt3lg* mRNA vs. control 'C' ($^{\&}p = 0.049$).

(G) Confocal microscope brain images of CD11c-eYFP mice (n = 3–6 mice per group). eYFP$^+$ cells (green) are located near reactive GFAP$^+$ astrocytes (red) at infarct periphery. Scale bar, 10 μm. Bars show mean ± SEM and symbols are values per mouse.

See also Figures S1 and S2.

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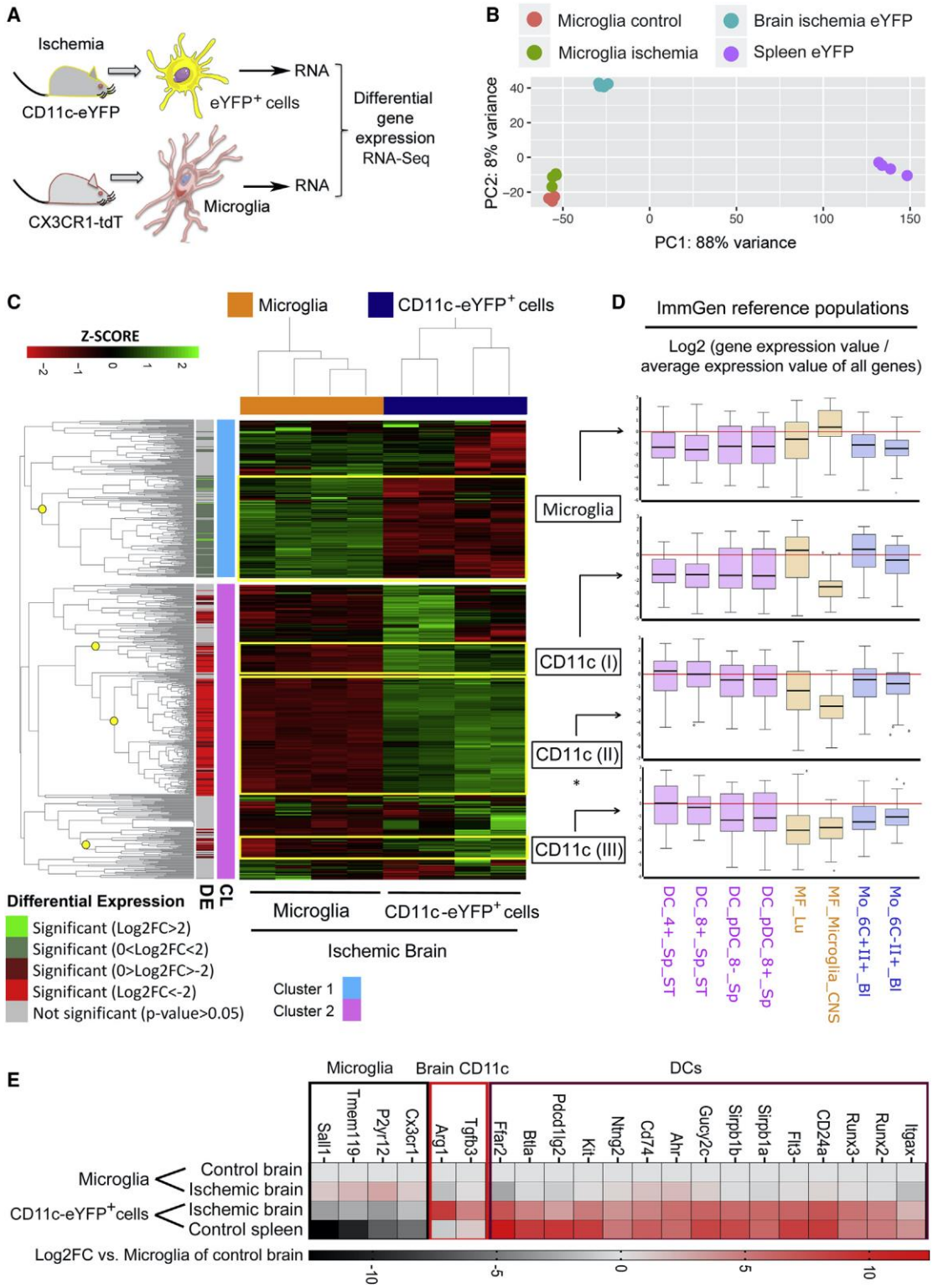

Figure 2. Differential Gene Expression between CD11c$^+$ Cells and Microglia of the Ischemic Mouse Brain

(A) RNA-seq of FACS-sorted microglia and eYFP$^+$ cells from CX3CR1cre^{ERT2}:Rosa26-tdT mice and CD11c-eYFP mice, respectively, 4 days post-ischemia. We also obtained microglia from control CX3CR1cre^{ERT2}:Rosa26-tdT mice and eYFP$^+$ cells from spleen of CD11c-eYFP control mice (n = 4 mice per group).

(B) Principal component analysis illustrates the unsupervised sample distribution.

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in each of these cell groups and showed a differential transcriptional signature in eYFP$^+$ cells versus microglia in the ischemic brain tissue. We found 202 pathways enriched in eYFP$^+$ cells compared with microglia but only 21 pathways enriched in microglia compared with eYFP$^+$ cells. The fact that only a few pathways were enriched in microglia versus eYFP$^+$ cells suggested that microglial genes could be expressed in the eYFP$^+$ population too. Functional annotation clustering (Table S1) highlighted functions overrepresented in eYFP$^+$ cells versus microglia, such as antigen presentation and immune responses (Figure S4C). Microglia displayed overrepresentation of genes related to neural functions, such as γ-aminobutyric acid neurotransmission, neuropeptides, and synaptic vesicles, among others, suggesting specific microglial-neuronal interactions that are less prominent or absent in CD11c$^+$ cells.

We then compared the transcriptomic data with reference cell population expression profiles defined by the ImmGen project (ImmGen Consortium, 2016; Miller et al., 2012) using differential gene clustering analysis. We selected eight myeloid cell reference populations defined by ImmGen (https://www.immgen.org/). We compared gene expression in those populations in a pairwise manner to identify genes with the highest differential expression in each ImmGen population. Then we compared the expression of those sets of genes in our samples (Figures 2C and 2D). To validate this process, we checked that our control samples showed correspondence to the appropriate ImmGen populations (Figure S5). Gene expression data in microglia and eYFP$^+$ cells of the ischemic brain (Figure 2C) were compared with the ImmGen populations (Figure 2D). Gene clustering analysis revealed subclusters of differentially expressed genes in our samples, with high representation among reference microglia, lung macrophages and Ly6C$^+$ monocytic blood population, and splenic DC populations.

For validation purposes, we selected several genes differentially expressed between CD11c$^+$ cells and microglia of the ischemic brain and conducted a qRT-PCR study in FACS-sorted eYFP$^+$ cells and microglia obtained from independent groups of mice (Figure 2E). eYFP$^+$ cells showed higher expression than microglia of typical DC genes, such as *Runx3*, *Runx2*, and *Kit*. Furthermore, expression of typical microglial markers was comparatively lower in the eYFP$^+$ cells. The selected group of genes clearly separated microglia genes from DC genes, as illustrated in the volcano plot (Figure S4D). Notably, among the selected genes, *Tgfb3* and *Arg1* were overrepresented only in CD11c$^+$ cells of the ischemic brain and not microglia or spleen CD11c$^+$ cells. Altogether the results suggest that the ischemic brain tissue displays a complex population of CD11c$^+$ cells, including cells with features of cDCs and cells with brain-specific features, which are distinctive from previously described canonical DC populations.

CD11c$^+$ Cells in the Ischemic Brain Include Microglia and Infiltrating DCs

We then characterized CD11c$^+$ cells by flow cytometry using the widely used definition of mouse microglia as CD45^{lo}CD11b$^+$ cells, distinct from peripheral myeloid cells that are CD45^{hi}CD11b$^+$ (Ford et al., 1995). Under steady-state conditions the majority of brain eYFP$^+$ cells were CD45^{lo}CD11b$^+$, with only a low proportion of CD45^{hi} cells (Figure 3A). In another group of control CD11c-eYFP mice, we dissected out the choroid plexus and meninges separating those regions from the brain parenchyma and studied the eYFP$^+$ cells using flow cytometry. Most eYFP$^+$ cells in the brain parenchyma were CD45^{lo}CD11b$^+$ microglial cells (95% $\pm$ 1%, n = 4 hemispheres, n = 2 mice), whereas eYFP$^+$ cells in choroid plexus and meninges were CD45^{hi} (43% $\pm$ 12%) or CD45^{lo} (25% $\pm$ 4%) (Figures 3B and 3C). Four days post-ischemia, the proportion of CD45^{hi} cells within brain eYFP$^+$ cells increased to about one half, whereas the other half corresponded to CD45^{lo}CD11b$^+$ cells (Figures 3D and 3E), showing a mixed population of brain resident and infiltrating CD11c$^+$ cells. CD45^{lo}CD11b$^+$eYFP$^+$ cells corresponded to 6.9% $\pm$ 2.2% (mean $\pm$ SD, n = 3) of the total CD45^{lo}CD11b$^+$ microglia in the injured brain hemisphere.

Infiltrating DCs Excel in Antigen Presentation Compared with Microglia

For functional information about CD11c$^+$ cells and microglia in the ischemic brain, we focused on antigen presentation. The expression of genes related to antigen presentation increased in the brain tissue after ischemia, as illustrated by the expression of *Cd74* mRNA encoding MHCII invariant chain, which peaked at day 4 (Figure 4A). The RNA-seq study showed that expression of MHCII genes was overrepresented in eYFP$^+$ cells compared with microglia in the injured tissue 4 days post-ischemia (Figure 4B, upper heatmap). Likewise, mRNA expression of MHCII complex transactivator *Ciita*, *Cd74*, and genes encoding immunomodulatory molecules, such as *Tnfsf4* (OX40L, CD252), *Dpp4*, *Btla*, and *Pdcd1lg2* (PD-L2), and the costimulatory molecules *Cd80* and *Cd40*, was higher in eYFP$^+$ cells than microglia (Figure 4B, lower heatmap). eYFP$^+$ cells were frequent in myelin-rich areas of the

(C and D) Comparison of transcriptomic data of ischemic microglia and eYFP$^+$ cells (C) with ImmGen reference cell population expression profiles using differential gene clustering analysis. Data represented as boxplots (D). Heatmap of differential gene expression between microglia and eYFP$^+$ cells of ischemic tissue (C). Unsupervised clustering analysis differentiated microglia from eYFP$^+$ cells by genes upregulated (green) or downregulated (red) in each group. We selected four subclusters (yellow squares in heatmap) by significance and magnitude (fold change) of their differential expression.

(D) Gene subclusters overrepresented in eYFP$^+$ cells (I, II, and III with 53, 203, and 34 genes, respectively) and one microglia subcluster (with 177 genes) were used for comparison with eight ImmGen reference populations: four types of spleen (Sp) DCs, lung (Lu) macrophages (MF), microglia, and blood (Bl) monocytes (Mo, Ly6C$^+$, and Ly6C$^-$). Asterisk indicates that because of limitations in the ImmGen My Geneset tool, only the 200 most differentially expressed genes (non-corrected p value < 0.01) of the CD11c (II) subcluster were included in the analysis.

(E) We selected 20 genes differentially expressed in eYFP$^+$ cells versus microglia for RT-PCR validation with microglia and eYFP$^+$ cells sorted from independent groups of ischemic mice (n = 4 per group). We grouped the genes according to their overrepresentation population as "Microglia," CD11c$^+$ cells (indicated as "DCs") in spleen and ischemic brain CD11c$^+$ cells, and "Brain CD11c" in ischemic brain CD11c$^+$ cells only. Multiple comparison t test between brain CD11c$^+$ versus microglia cells showed statistically significant differences for all genes (Holm-Sidak, α = 0.05).

See also Figures S3–S5 and Table S1.

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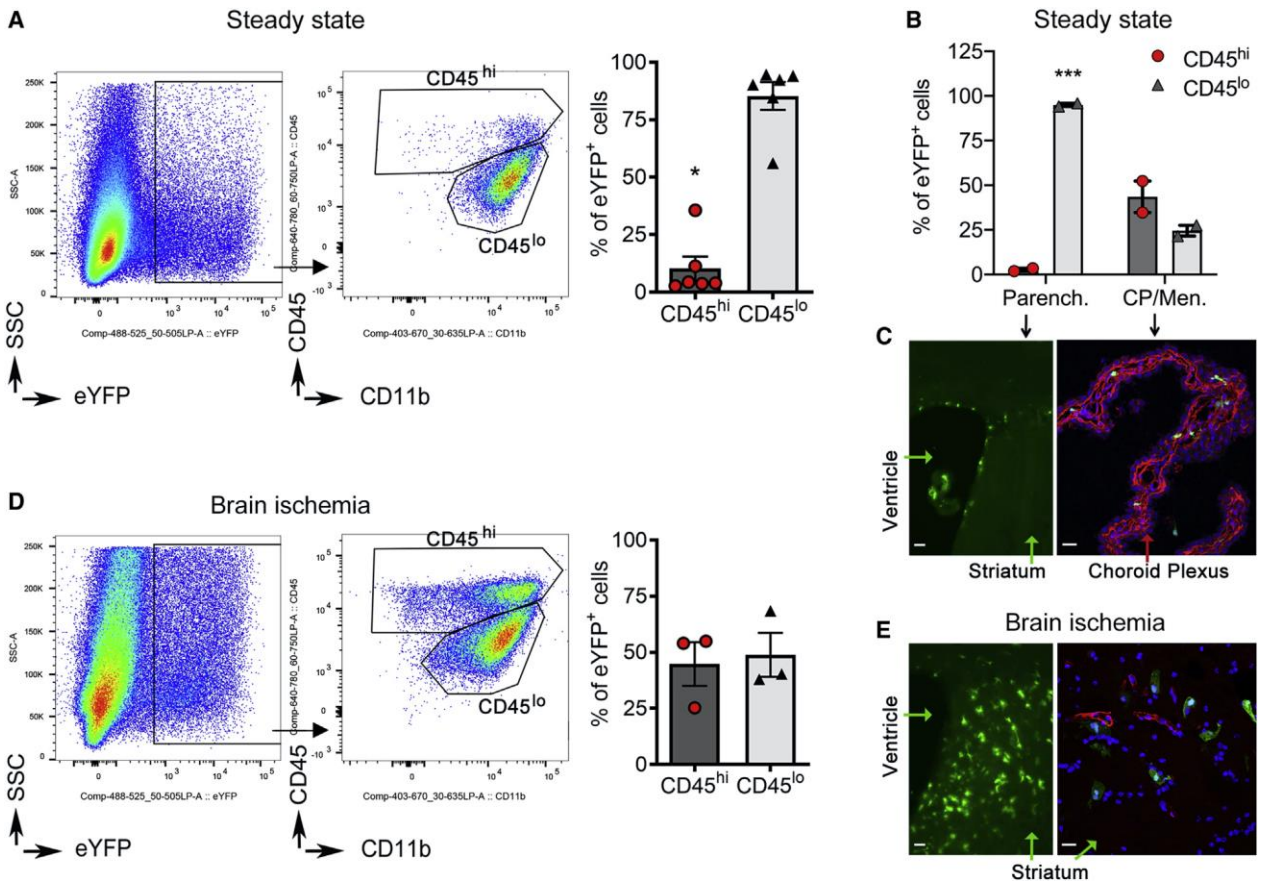

Figure 3. CD11c$^+$ Cells in the Inflamed Brain Parenchyma Include Microglia and Infiltrating DCs

(A) Most eYFP$^+$ cells are CD45^{lo}CD11b$^+$ at steady state (Wilcoxon matched-pairs signed rank test, *p = 0.03, n = 6 eYFP-CD11c mice).

(B) Separation of choroid plexus and meninges (CP/Men.) from brain parenchyma (Parench.) confirmed that most eYFP$^+$ cells in brain parenchyma were CD45^{lo}CD11b$^+$ cells (n = 2 mice, two brain hemispheres processed separately for each mouse; two-way ANOVA, ***p = 0.0003), whereas CD45^{hi}CD11b$^+$eYFP$^+$ cells were found in choroid plexus and meninges.

(C) Images of eYFP$^+$ cells (green) in each region shown in (B).

(D) Flow cytometry of brain tissue 4 days post-ischemia shows that approximately half of the brain eYFP$^+$ cells are CD45^{hi} and the other half are CD45^{lo}CD11b$^+$ microglia (n = 3 mice).

(E) Representative images of ischemic tissue show abundant eYFP$^+$ cells (green) in parenchyma. Images on the right-hand side in (C) and (E) are immunostained with pan-laminin (red) and nuclei (To-Pro3, blue). In graphs, bars are mean ± SEM, and symbols show individual values per mouse. Scale bar, 20 μm.

injured tissue, where they may interact with myelinated nerve fibers (Figure 4C). Among the CD11c$^+$ cell population, CD45^{hi} cells expressed notably higher levels of MHCII than CD45^{lo}CD11b$^+$ microglia (Figure 4D), suggesting that the subset of CD11c$^+$ microglia has limited capacity to present antigen through MHCII.

We then asked whether antigen presentation capacity was different in CD11c$^+$ cells after separating between CD45^{hi} and CD45^{lo} cells. CD80 expression was rather low (<1% of the respective parent populations; data not shown). However, approximately 20% of CD45^{hi}CD11c$^+$ cells expressed the immunostimulatory molecule OX40L, whereas fewer than 5% of CD45^{lo}CD11c$^+$ microglia did, and we did not detect this molecule in CD45^{lo}CD11c$^-$ microglia (Figure 4E). Among the CD45^{hi}CD11b$^+$CD11c$^+$ cells, we detected the presence of a major population of cDC2 (CD172$^+$) and a smaller population of cDC1 cells (XCR1$^+$) (Figure 4F; Figure S6). cDC2 were positive for immunomodulatory molecule OX40L, whereas cDC1 were not (Figure 4G).

For a functional assessment, we studied the capacity of CD45^{lo} cells, including CD11c$^-$ and CD11c$^+$ microglia, and CD45^{hi}CD11c$^+$ cells sorted from the ischemic brain tissue to induce T cell proliferation. To this end, we used OTII transgenic mice with CD4$^+$ T cells specific for ovalbumin (OVA) 323–339 peptide. We co-cultured these CD4$^+$ T cells in the presence or absence of CD45^{lo}CD11b$^+$eYFP$^-$ microglia, CD45^{lo}CD11b$^+$eYFP$^+$ microglia, or CD45^{hi}CD11b$^+$eYFP$^+$ cells sorted from the brain of CD11c-eYFP mice 4 days after ischemia and exposed to OVA peptide (chicken OVA 323–339 peptide). Irrespective of CD11c expression, microglia showed a reduced capacity to stimulate T cell proliferation compared with CD45^{hi}CD11b$^+$eYFP$^+$ cells (Figure 4H). For class I-restricted

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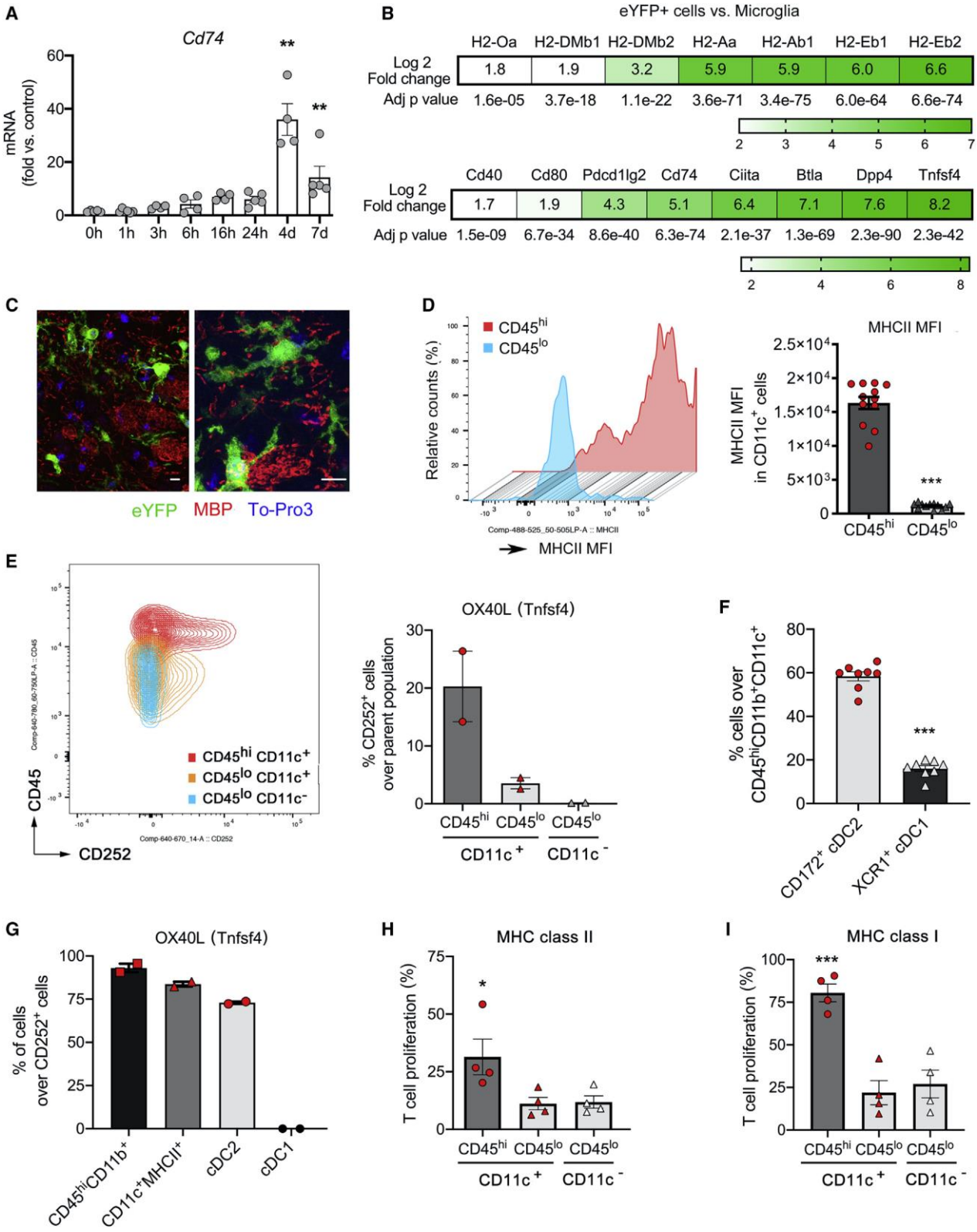

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antigen presentation, we used OTI transgenic mice with CD8$^+$ T cells specific for the OVA 257–264 peptide (SIINFEKL). We obtained CD8$^+$ T cells from these mice and co-cultured them with CD45^{lo}CD11b$^+$eYFP$^-$ microglia, CD45^{lo}CD11b$^+$eYFP$^+$ microglia, or CD45^{hi}CD11b$^+$eYFP$^+$ cells (Figure S7) previously exposed to a pulse of SIINFEKL peptide. Again, CD45^{hi} CD11b$^+$eYFP$^+$ cells were the most efficient population to induce CD8$^+$ T cell proliferation (Figure 4I). Therefore, the expression of CD11c in a subset of microglia of the adult brain does not confer a higher capacity to induce adaptive immune responses, while infiltrating CD45^{hi}CD11b$^+$eYFP$^+$ cells represent a bona fide DC population of cells that excel in their antigen presenting capacity.

Selective Characterization of Infiltrating DCs with Parabiosis Studies

To identify CD11c$^+$ cells trafficking from the periphery to the inflamed brain we generated PA pairs by joining the circulation of wild-type (WT) mice and transgenic CD11c-eYFP littermates (Figure 5A). After 3 weeks of parabiosis, we detected a few eYFP$^+$ cells located mainly in the leptomeningeal zone and choroid plexus, while being absent from the brain parenchyma (Figures 5Bi–5Biii). Therefore, the choroid plexus/meninges contain a dynamic CD11c$^+$ population of cells capable of interchanging with the periphery in steady state. In contrast, CD11c$^+$ cells located in the brain parenchyma are a brain-resident population. In other groups of PA mice, we induced cerebral ischemia in the WT mouse of each PA pair (Figure 5A). Four days post-ischemia, we detected eYFP$^+$ cells in the ischemic brain parenchyma (Figure 5Biv–5Bvii), leptomeninges, and choroid plexus (Figure 5Bvi) of the PA WT mice. Flow cytometry (Figure S8) confirmed the very small number of eYFP$^+$ cells in the brain of the PA WT mice in steady state and the increased number following ischemia (Figure 5C). Most infiltrating eYFP$^+$ cells were CD45^{hi}CD11b$^+$ cells (mean ± SD 84.3% ± 4.4%, n = 5).

We then compared the transcriptomic profile of brain eYFP$^+$ cells sorted from the PA ischemic WT mice (infiltrating eYFP$^+$ cells) with that of microglia sorted from the brain of ischemic mice. In parallel, we sorted eYFP$^+$ cells from the brain of ischemic CD11c-eYFP mice (total brain eYFP$^+$ cells) for comparative purposes. Principal component analysis separated infiltrating cells (PA eYFP) from microglia and from total eYFP$^+$ cells (Figure 5D). Comparison of the transcriptomic data of our cells with that of ImmGen reference cell populations (Figure 5E) showed a subcluster of genes similar to lung macrophages, and subclusters with similarities to spleen DCs and Ly6C$^+$ blood monocytes. The top 50 genes overrepresented in PA eYFP$^+$ cells versus microglia in the ischemic brain are shown in Table S2. Infiltrating PA eYFP$^+$ cells showed certain differences versus microglia in the expression of genes encoding C-type lectin receptors (CLRs) and endosomal Toll-like receptors (TLRs). A wide repertoire of pattern recognition receptors (PRRs) of the CLR family, including MCL (*Clec4d*), DNGR-1 (*Clec9a*), DCAR (*Clec4b1*), MINCLE (*Clec4e*), DECTIN-2 (*Clec4n*), CD301 (*Clec10a*), CD371 (*Clec12a*), OCILRP2 (*Clec2i*), ZNF705A (*Clec4a1*), and DECTIN-1 (*Clec7a*) were overrepresented in infiltrating PA eYFP$^+$ cells versus microglia (Figure 5F). In contrast, microglia overexpressed brain-associated C-type lectin (BACL, *Clec2l*), whose expression is high in brain tissue (Lysenko et al., 2013). Expression of *Tlr7*, *Tlr3*, and *Tlr12* was higher in microglia, whereas PA eYFP$^+$ cells showed upregulation of *Tlr8*. These results illustrate cellular specialization in innate sensing and show a better overall equipment in infiltrating DCs. Enriched pathways in PA eYFP$^+$ cells versus microglia highlighted immune functions in the former (Table S3). Enrichment analysis identified overrepresentation of functions related to neuronal development and differentiation, as well as terms and pathways related to cell proliferation, in ischemic microglia compared with eYFP$^+$ cells of PA ischemic WT mice (Table S3). Accordingly, we did not detect Ki67$^+$ eYFP$^+$ cells in the ischemic brain of PA WT mice (data not shown), whereas we found it after ischemia in non-PA CD11c-eYFP mice (see Figure S2B). Gene set enrichment analysis (GSEA) also identified overrepresentation in microglia of genes involved in the cell division cycle, including G2/M checkpoint, mitotic spindle assembly, cell cycle-related targets of E2F transcription factors, and genes associated with spermatogenesis (including genes with neuroendocrine secretory functions and genes involved in cell proliferation) (Figure 5G; Figure S9). Moreover, GSEA highlighted an IFN-α signature induced by ischemia

Figure 4. Infiltrating CD11c$^+$ Cells Surpass Microglia in Antigen Presentation Capacity

(A) MHCII antigen-associated *Cd74* mRNA expression in brain tissue at different time points (from immediately after ischemia (0 h) to 7 days; n = 3–7 mice per time point, Kruskal-Wallis test and Dunn's multiple-comparisons test, **p < 0.01).

(B) Differential gene expression between eYFP$^+$ cells and microglia sorted from ischemic brain shows overrepresentation of genes involved in antigen presentation, co-stimulation, and immunomodulation in eYFP$^+$ cells.

(C) eYFP$^+$ cells (green) in myelin-rich areas (MBP, red) 7 days post-ischemia. Nuclei are stained with To-Pro3 (blue). Scale bar, 10 μm.

(D) Among CD11c$^+$CD11b$^+$ cells in ischemic brain, CD45^{hi} cells display higher MHCII expression than CD45^{lo} cells, as showed by the MHCII mean fluorescence intensity (MFI) (paired t test, ***p < 0.001, n = 12 mice).

(E) OX40L (CD252, *Tnfsf4*) expression is high in eYFP$^+$CD45^{hi} DCs, but not CD45^{lo} microglia 4 days post-ischemia (n = 2 mice).

(F) CD45^{hi}CD11b$^+$CD11c$^+$ cells 4 days post-ischemia comprise different subsets of DCs, including CD172$^+$ cDC2 cells and XCR1$^+$ cDC1 cells (Wilcoxon matched-pairs signed rank test, **p = 0.008, n = 8 mice).

(G) cDC2 cells express OX40L, whereas cDC1 do not.

(H) Proliferation of OTII T cells stimulated with OVA peptide 323–339 in the presence of either eYFP$^+$CD45^{hi} DCs, eYFP$^+$CD45^{lo}CD11b$^+$ microglia, or eYFP$^-$ CD45^{lo}CD11b$^+$ microglia obtained by FACS from the brain of CD11c-eYFP mice 4 days post-ischemia (n = 4 mice). One-way ANOVA with a repeated-measure design and Holmes-Sidak multiple-comparisons test showed higher T cell proliferation induced by CD45^{hi}CD11c$^+$ DCs versus CD11c$^+$ microglia (*p = 0.032) or CD11c$^-$ microglia (*p = 0.028).

(I) Proliferation of OTI CD8 T cells exposed to cells (obtained as in H) that were previously pulsed with SIINFEKL peptide. Again CD45^{hi}CD11c$^+$ cells showed higher capacity than microglia to induce CD8$^+$ T cell proliferation. Statistical analysis as in (G), ***p < 0.0001 for both CD11c$^+$ and CD11c$^-$ microglia, n = 4 experiments performed with cells obtained from different ischemic mice. Bars show mean ± SEM and symbols show individual values per each mouse.

See also Figures S6 and S7.

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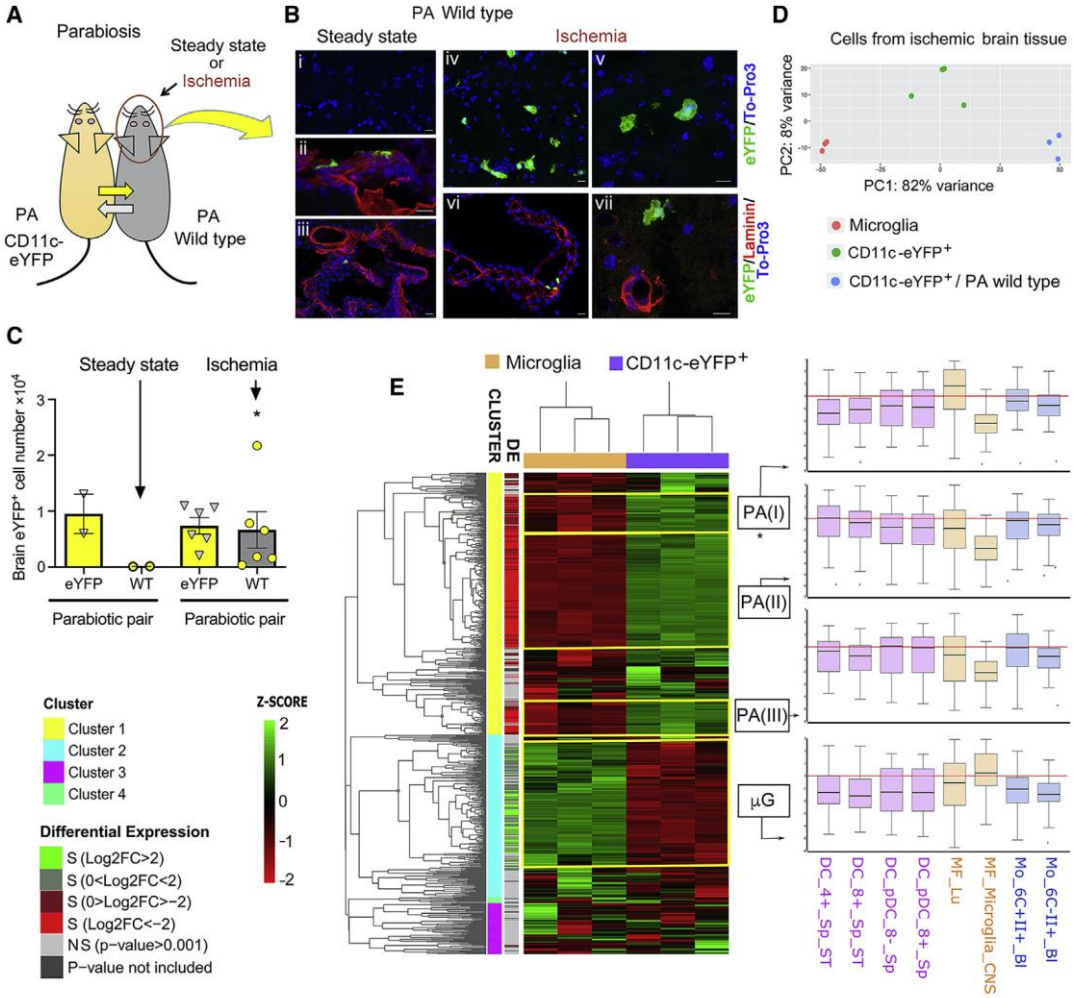

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	Clec	2l	7a	4a1	2i	12a	10a	4n	4e	4b1	9a	4d
PA CD11c-eYFP > Microglia		-4.8	1.5	2.1	3.4	3.6	3.7	3.8	4.2	4.2	5.3	5.6
Adj p value		1.7e-10	1.7e-17	8.0e-6	8.3e-22	1.5e-11	1.3e-75	4.2e-14	3.6e-6	1.7e-9	7.1e-10	8.7e-11

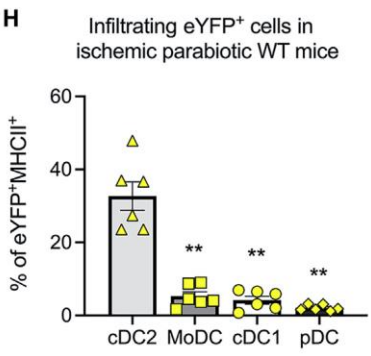
Log 2 (Fold change): -5, 0, 5

	Tlr12	Tlr3	Tlr7	Tlr8
PA CD11c-eYFP > Microglia	-3.2	-2.6	-1.8	3.3
Adj p value	1.6e-06	8.1e-08	8.7e-15	2.8e-41

Log 2 (Fold change): -4, -2, 0, 2, 4

G

	NAME	SIZE	NES	NOM p-val	FDR q-val	RANK AT MAX
PA eYFP+	ROS PATHWAY	47	0.698	0.0	0.0	2860
	OXIDATIVE_PHOSPHORYLATION	196	0.588	0.0	0.0	4470
	CHOLESTEROL_HOMEOSTASIS	73	0.482	0.0	0.024	1869
	IL2_STAT5_SIGNALING	193	0.458	0.00	0.035	1435
	ANGIOGENESIS	33	0.441	0.04	0.040	1046
Microglia	INTERFERON_ALPHA_RESPONSE	90	-0.564	0.0	0.007	3315
	G2M_CHECKPOINT	193	-0.551	0.0	0.004	3184
	MITOTIC_SPINDLE	198	-0.505	0.0	0.010	2539
	E2F_TARGETS	196	-0.500	0.0	0.009	3763
	TGF_BETA_SIGNALING	53	-0.450	0.02	0.029	1975
	SPERMATOGENESIS	110	-0.421	0.0	0.047	2146



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in microglia only, and it identified upregulation of TGF-β signaling in microglia but not in infiltrating eYFP$^+$ cells (Figure 5G; Figure S9). In contrast, features of the infiltrating eYFP$^+$ cells included an active response to reactive oxygen species, high oxidative and lipidic metabolism, STAT5-dependent signaling, and angiogenesis (Figure 5G; Figure S9).

We then studied the identity of peripheral eYFP$^+$ cells infiltrating the ischemic brain tissue of WT PA mice by phenotyping DC subsets by flow cytometry. Infiltrating eYFP$^+$ cells included a major population of CD172$^+$ cDC2, and less abundant XCR1$^+$ cDC1, pDCs, and monocyte-derived DCs (moDCs) (Figure 5H; gating strategy is shown in Figure S8).

Chemoattraction of DCs to the Ischemic Brain

We investigated the signals that may attract peripheral DCs to the inflamed brain. The RNA-seq analysis of parabionts showed high overrepresentation of several genes encoding chemokine receptors in infiltrating eYFP$^+$ cells versus microglia of ischemic mice (Figure 6A). Of note, only the expression of atypical chemokine receptor 3 (*Ackr3* encoding for CXCR7) was higher in microglia than eYFP$^+$ cells (Figure 6A). We validated some of these chemokine receptor genes by RT-PCR in RNA of FACS-sorted cells obtained from independent groups of mice (Figure 6B). Notably, ischemia induced mRNA expression of chemokines binding those DC receptors at different time points (Figure 6C; Figure S10A). *Ccl5* and *Ccl8* mRNA expression was strongly induced 4 and 7 days post-ischemia. This time course was compatible with the time point of CD11c$^+$ cell accumulation (see Figure 1C). We isolated microglia, astrocytes, and endothelial cells from control brain tissue and 4 days post-ischemia to identify which brain cells express these chemokines. Ischemia upregulated *Ccl8* and *Ccl5* expression mainly in microglia (Figure 6D). To find out whether microglia contributed to DC infiltration to the ischemic brain tissue, we used a pharmacological strategy to deplete microglia. Given that microglial cell viability is CSF1R dependent, chronic treatment with the CSF1R inhibitor PLX5622 provided in the diet causes strong microglial depletion (Otxoa-de-Amezaga et al., 2019). The treatment reduced CD45^{hi}CD11c$^+$ cells expressing MHCII (i.e., infiltrating DCs), while it did not modify CD11c$^+$MHCII$^+$ cell numbers in the spleen (Figure 6E). Microglia depletion reduced the numbers of all DC subsets as follows: cell number values (mean ± SD) for PLX5622 diet versus control diet, respectively (n = 12 per group, Mann-Whitney test) are for cDC1, 370 ± 282 versus 1,509 ± 818 ($p = 0.007$); for cDC2, 8,223 ± 3,776 versus 18,277 ± 11,209 ($p = 0.012$); for moDCs, 960 ± 670 versus 1,869 ± 1,136 ($p = 0.033$); and for pDCs, 1,638 ± 1,306 versus 2,811 ± 1,054 ($p = 0.014$). This effect was attributable to the reduction of chemokine production in the ischemic brain tissue after microglia depletion (Figure 6F). cDC2, moDC, and pDC subsets were similarly affected by the absence of microglia, as the percentage over the parent population of CD11c$^+$MHCII$^+$ cells was similar between microglia-depleted and non-depleted groups. Of note, cDC1 showed a stronger reduction than the other DC subsets after microglia depletion (Figure 6E). Therefore, cDC1 recruitment to the ischemic brain appears to be particularly dependent on chemokines generated by microglia.

cDC1 Exert Protective Functions in Brain Ischemia

CCL8, CCL5, and CCL3 bind CCR1, suggesting that this receptor expressed in brain infiltrating DCs (see Figures 6A and 6B) could be involved in the migration of CD11c$^+$ cells to the

Figure 5. Characterization of Infiltrating CD11c$^+$ Cells Obtained from Parabiotic Mice

(A) Parabiotic (PA) pairs obtained by joining CD11c-eYFP mice with wild-type (WT) littermates. After 3 weeks we studied the brain of each pair (n = 4), or we induced brain ischemia in the WT mouse of each pair and studied the brain 4 days later (n = 11).

(B) Brain tissue immunofluorescence of PA WT mice in steady state (i–iii) (n = 2) and after ischemia (iv–vii) (n = 2) show eYFP$^+$ cells (green). Nuclei are stained with To-Pro3 (blue). Images (ii, iii, vi, and viii) are also stained with pan-laminin (red). In steady state, there are few eYFP$^+$ cells in the subpial space (ii) and choroid plexus (iii) but not in brain parenchyma (i) of the WT parabiont. eYFP$^+$ cells in meninges, choroid plexus (vi) and ischemic brain parenchyma (iv, v, and vii) of the ischemic WT parabiont. Scale bar, 10 μm.

(C) eYFP$^+$ cell number (flow cytometry) in brain of PA pairs. At steady state, the number of eYFP$^+$ cells (mean ± SD) in WT PA brain is small (117 ± 41 eYFP$^+$ cells, n = 2). Ischemia increases eYFP$^+$ cells in PA-WT brain (6,638 ± 7,955 eYFP$^+$ cells, n = 6) ($p = 0.03$, one-sample Wilcoxon test).

(D) Transcriptome analysis of eYFP$^+$ cells sorted from the ischemic brain of PA-WT mice (n = 3) or ischemic CD11c-eYFP mice (n = 4) and microglia (n = 3 per group). Principal component analysis shows sample distribution.

(E) Heatmap representation of differential genes upregulated (green) or downregulated (red) among microglia and infiltrating eYFP$^+$ cells from the PA-WT mice using 776 genes retrieved from the comparison with ImmGen reference cell populations. The representation includes 4 natural clusters found by unsupervised clustering: subclusters PA(I), PA(II), and PA(III) were upregulated in PA samples, and subcluster μG was upregulated in microglia. Subclusters contained 62, 185, 55, and 200 genes, respectively. The expression of genes included in each subcluster was checked on the ImmGen reference cell populations, and the generated boxplots are displayed in the figure. *This subcluster was generated by merging together the genes contained in three adjacent subclusters because of the small number of genes and similar expression profile in reference populations. Data in graphs are expressed as $\log_2$ (gene expression value/average expression value of all genes).

(F) Differential gene expression between CD11c-eYFP$^+$ cells sorted from PA WT mice and microglia after ischemia illustrates overrepresentation of genes encoding C-type lectin receptors (Clec) in infiltrating eYFP$^+$ cells versus microglia, with the exception of Clec2l higher in microglia. Endocytic TLRs also showed some differential expression between these cells.

(G) Table showing GSEA results. The top rows with positive enrichment score correspond to PA eYFP$^+$ cells and the bottom rows with negative enrichment score correspond to microglia. Only the pathways with a false discovery rate (FDR) of less than 5% are shown. "Size" is the number of gene sets enriched in each phenotype; NES, normalized enrichment score; NOM p-val, nominal p value; RANK AT MAX, position in the ranked list at which the maximum enrichment score occurred; ROS, reactive oxygen species.

(H) Flow cytometry of subpopulations of eYPF$^+$ DCs infiltrating the brain of ischemic WT mouse of each parabiotic pair (n = 6 pairs). Numbers of eYFP$^+$ cDC2 cells are higher than eYFP$^+$ cDC1 cells (**$p = 0.004$), eYFP$^+$ moDCs (**$p = 0.006$), and eYFP$^+$ pDCs (**$p = 0.002$) (one-way ANOVA with a repeated-measures design and Dunnett's multiple-comparisons test). Bars show group mean ± SEM and points are values per mouse.

All mice used in this figure were females. See also Figures S8 and S9 and Tables S2 and S3.

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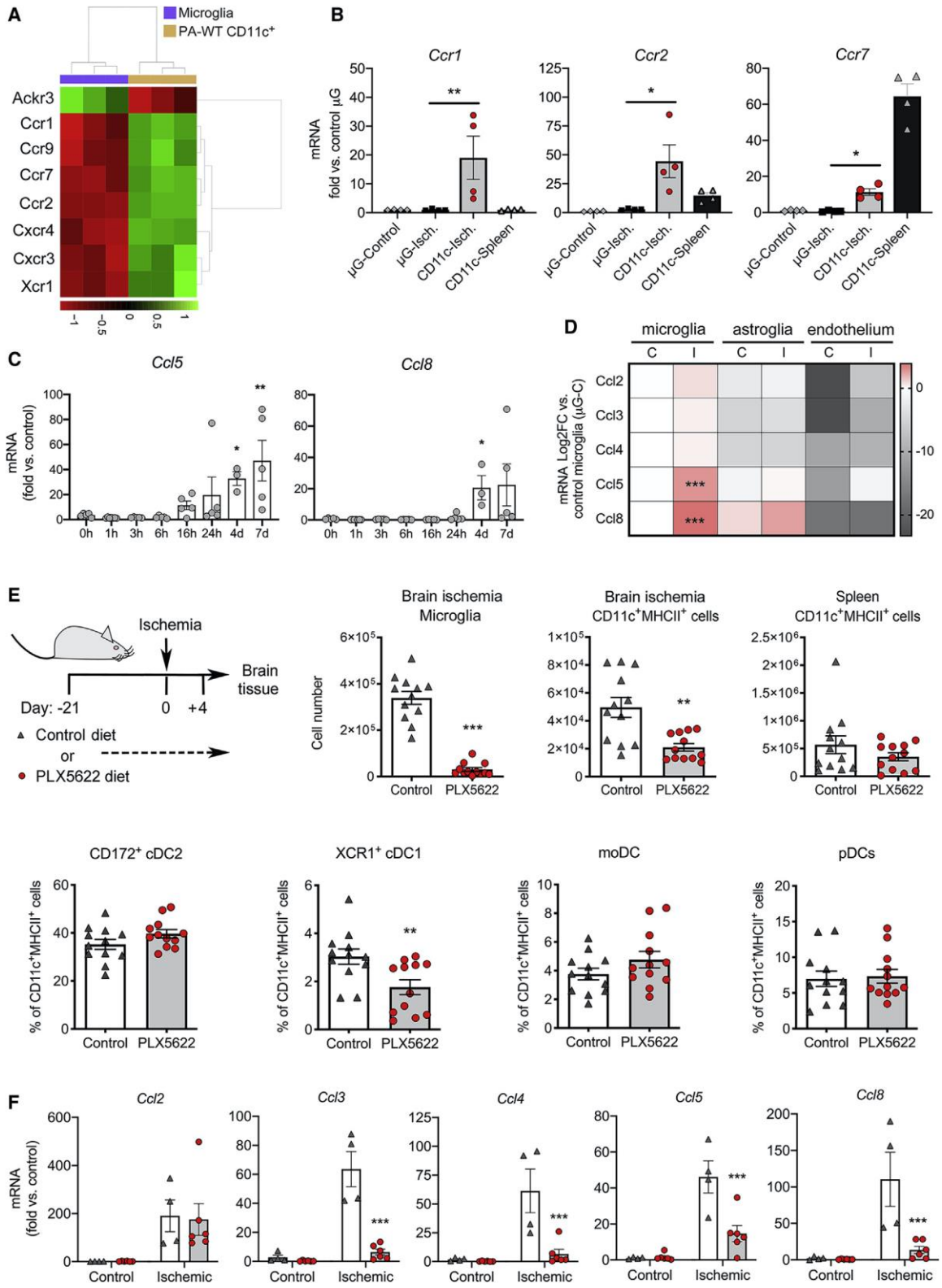

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ischemic tissue. To test this possibility, we blocked CCR1 by systemic (intraperitoneal [i.p.]) administration of CCR1 antagonist J113863 (Amat et al., 2006) to WT mice (Figure 7A). The drug did not induce significant changes in infiltrating leukocytes, pDCs, moDCs, and CD172$^+$ cDC2 cells but reduced XCR1$^+$ cDC1 cell number (Figure 7B; Figure S10B). The neurological score and the sizes of the lesion were not different between groups before treatment, as assessed by MRI and behavioral tests 24 h post-ischemia, prior to drug administration. Drug treatment did not modify the size of the lesion versus the vehicle 4 days post-ischemia (Figure S10C). However, the neurological score worsened from day 1 to day 4 in the CCR1 antagonist group versus the vehicle group (Figure 7C), suggesting a beneficial effect of cDC1 in brain ischemia. cDC1 cell development requires the transcription factor BATF3 (Hildner et al., 2008). cDC1 cells are strongly reduced in *Batf3*$^{-/-}$ mice, and we did not detect these cells in the ischemic tissue of *Batf3*$^{-/-}$ mice (Figure 7D), whereas the other DC populations were not significantly affected (Figure 7D; Figure S10D). *Batf3*$^{-/-}$ mice showed larger infarctions and worse neurological deficits than WT mice (Figure 7E).

We verified that worse stroke outcome in *Batf3*$^{-/-}$ mice was not attributable to alterations in cerebrovascular anatomy such as the extent of collateral circulation (Figure S10E). To find out whether infiltrating hematopoietic cells contributed to the worse outcome of *Batf3*$^{-/-}$ mice, we generated chimeric mice by transplanting bone marrow of either *Batf3*$^{-/-}$ mice or WT (*Batf3*$^{+/+}$) mice to WT recipient mice (Figure 7F). After ischemia, lesion volume slightly increased from day 1 to day 4 in chimeric mice with *Batf3*$^{-/-}$ hematopoietic cells but not in mice with WT hematopoietic cells (Figure 7G). Accordingly, at day 4 post-ischemia, neurological function was worse in the mice with *Batf3*$^{-/-}$ hematopoietic cells (Figure 7G). Altogether these results support a beneficial effect of infiltrating cDC1s in brain ischemia.

DISCUSSION

We report that most CD11c$^+$ cells found in the mouse brain parenchyma in steady state are CD45^{lo}CD11b$^+$ microglia, whereas the choroid plexus and meninges display CD45^{hi}CD11c$^+$ cells that traffic from the periphery. CD11c$^+$ cells in the ischemic tissue originate from a complex mixture of DCs invading the inflamed brain and proliferating resident microglia, in line with a previous study (Felger et al., 2010). Our study further demonstrates that CD11c$^+$ microglia differ from DCs in the limited capacity of the former to present antigen and induce T cell proliferation. The population of microglial cells is heterogeneous and shows phenotypic and functional diversity (Silvin and Ginhoux, 2018). A CD11c$^+$ microglial subset is involved in myelinogenesis (Wlodarczyk et al., 2017), and several lines of evidence support specific functions and protective effects of CD11c$^+$ microglia (Benmamar-Badel et al., 2020). In our study both microglia and infiltrating CD11c$^+$ cells expressed genes involved in functions related to glial development and differentiation, whereas neuronal function was represented in microglia.

Necrotic cell death induced by ischemia generates danger-associated molecular patterns (DAMPs) and activation of innate immune receptors (Iadecola and Anrather, 2011). DAMPS can induce sterile inflammation through activation of various PRRs, including TLRs and certain CLRs (Gong et al., 2020). Immature DCs are tolerogenic, but they mature to immunostimulatory phenotypes after PRR activation. Brain infiltrating DCs showed a different CLR and TLR expression repertoire than microglia, suggesting cellular specialization in the recognition of danger signals in the injured brain. The transcriptomic analysis also showed overrepresentation of several chemokine receptors, such as *Ccr1* and *Ccr2*, in infiltrating CD11c$^+$ cells versus microglia, whereas only *Ackr3* expression was higher in microglia than infiltrating CD11c$^+$ cells. ACKR3, a scavenger of CXCL12, was previously found in microglia in which expression increased under inflammatory stimuli (Lipfert et al., 2013). Microglial cells seem to be instrumental for attraction of peripheral DCs to the inflamed brain by upregulating the expression of *Ccl5* and *Ccl8* mRNA. Accordingly, microglia depletion reduced ischemia-induced expression of chemokines in brain and attenuated DC infiltration, particularly of cDC1. Therefore, glial cells are critical for recruiting and maintaining DCs in the inflamed brain, as microglia generate DC chemoattractants and astrocytes produce Flt3L necessary to support DC viability (Coates et al., 2003; Guermonprez et al., 2013). Microglia depletion strongly reduced DC

Figure 6. CCR1-Mediated Chemoattraction of cDC1s

(A) Chemokine receptor genes overrepresented ($p < 0.001$, $\log_2$ fold change [FC] > 2) in infiltrating eYFP$^+$ cells of parabiotic (PA) WT ischemic mice versus microglia of ischemic mice (n = 3 mice per group).

(B) Validation by RT-PCR in microglia from control and ischemic brain (Isch; 4 days post-ischemia), CD11c$^+$ cells from ischemic brain, and spleen CD11c$^+$ cells (n = 4 per group). Kruskal-Wallis test and Dunn's multiple-comparisons test, $^*p < 0.05$ and $^{**}p < 0.01$.

(C) Brain *Ccl5* and *Ccl8* mRNA expression at different time points after ischemia (n = 3–7 mice per time point). Kruskal-Wallis test and Dunn's multiple-comparisons test, $^*p < 0.05$ and $^{**}p < 0.01$.

(D) Chemokine mRNA expression (RT-PCR) in microglia, astroglia, and endothelial cells sorted from control (C) or ischemic (I) brain (day 4) (n = 4 per group). Values are expressed as $\log_2$ fold change versus control microglia. Ischemia increased *Ccl8* and *Ccl5* mRNA expression mainly in microglia. Multiple-comparison t test, $^{***}p < 0.001$.

(E) WT mice received either diet with CSF1R inhibitor PLX5622 or control diet for 25 days (n = 12 mice per group). After 21 days of treatment, ischemia was induced, and brain was studied 4 days later using flow cytometry. PLX5622 severely depleted microglia and reduced the number of CD11c$^+$MHCII$^+$ infiltrating DCs in the ischemic brain but did not alter the number of spleen DCs. Mann-Whitney test, $^{***}p < 0.001$ and $^{**}p = 0.002$. Microglia depletion specifically reduced the recruitment of cDC1 cells to ischemic tissue (Mann-Whitney test, $^{**}p = 0.007$).

(F) Chemokine mRNA expression at day 4 in ischemic brain (striatum) of PLX5622-treated mice (n = 6) or controls (n = 4) (symbols as in 'E'). Although microglia depletion reduced the expression of *Ccl3* ($p < 0.0001$), *Ccl4* ($p = 0.0003$), *Ccl5* ($p = 0.0003$), and *Ccl8* ($p = 0.0006$) mRNA, it did not modify *Ccl2* mRNA ($p = 0.970$). Two-way ANOVA by treatment and ischemia effects, followed by Sidak's multiple-comparisons test; control tissue is corresponding contralateral non-ischemic hemisphere. Bars show group mean ± SEM and points are values per mouse.

See also Figure S10A.

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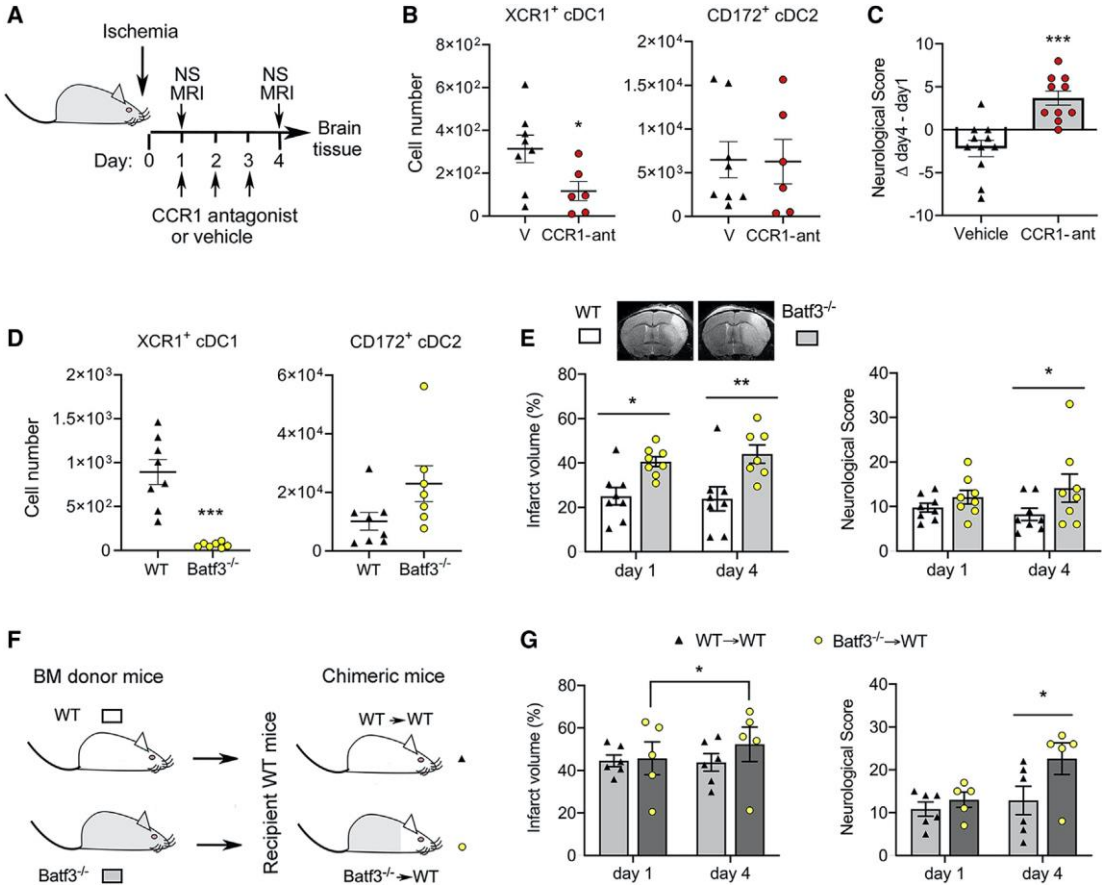

Figure 7. Protective Role of cDC1 in Brain Ischemia

(A) Daily administration of CCR1 antagonist J113863 (CCR1-ant) or the vehicle starting 1 day post-ischemia until day 3. We monitored the brain lesion using MRI and assessed the neurological function with a neuroscore (NS) (higher values indicate worse deficits). Brain tissue was studied at day 4 using flow cytometry.

(B) CCR1-ant (n = 6) did not affect cDC2 number but reduced cDC1 (n = 8) (Mann-Whitney test, ***p = 0.0007).

(C) Prior to drug treatment, the neurological score (mean ± SD; vehicle, 11.3 ± 5.0; CCR1-ant, 10.0 ± 5.1) was similar in both groups. Four mice per group died and were excluded. The neurological score worsened from day 1 to day 4 in the CCR1-ant group (n = 10) versus the vehicle group (n = 11) (Mann-Whitney test, ***p = 0.0002). "Δ day 4–day1" is the difference in NS between the two time points in the same mice.

(D and E) We induced ischemia in *Batf3*$^{-/-}$ mice (n = 8) and WT mice (n = 8) and studied the brain 4 days later (D). Batf3 deficiency depleted cDC1 cells and (E) increased MRI infarct volume. Two-way ANOVA by genotype and time point with a repeated-measures design; genotype effect, p = 0.005; Sidak's multiple-comparisons test showed significant differences between genotypes at both time points (*p = 0.018 and **p = 0.002). The NS was also worse in *Batf3*$^{-/-}$ mice than WT mice. Friedman test followed by Dunn's multiple-comparisons test, *p = 0.031.

(F) We generated chimeric mice by transplanting bone marrow (BM) of either *Batf3*$^{-/-}$ (n = 5) or WT (n = 6) donor mice to recipient WT mice. Chimeric mice with *Batf3*$^{-/-}$ BM lacked cDC1 cells in the spleen (<0.3% cDC1 over CD11c^{+}MHCII^{+} cells) compared with the mice with WT BM (10%–15% cDC1).

(G) After ischemia, mice with *Batf3*$^{-/-}$ BM show an increase in infarct volume from day 1 to 4 that is not detected in mice with WT BM (two-way ANOVA with repeated-measures design followed by Sidak's multiple-comparisons test, *p = 0.047). Accordingly, the NS is worse in the mice with *Batf3*$^{-/-}$ BM 4 days post-ischemia (Mann-Whitney test, *p = 0.037). Bars show group mean ± SEM and points are values per mouse.

See also Figures S10B–S10E.

infiltration but did not entirely abrogate it. It is plausible that other brain cells such as astrocytes or endothelial cells could participate in attracting DCs.

Different subsets of DCs display various immunomodulatory mechanisms to interact and shape T cell responses (Bourque and Hawiger, 2018). Compared with microglia, infiltrating CD11c^{+} cells overexpressed genes of typical co-stimulatory molecules *Cd80*, *Cd40*, and *Dpp4* (CD26) (Hatano et al., 2015; Ohnuma et al., 2008), and the immunomodulatory gene *Tnfsf4* (OX40L or CD252). OX40L binds OX40 on T cells to stimulate clonal expansion of effector and memory T cells (Croft et al., 2009), and it can induce regulatory T cell dysfunction (Jacquemin et al., 2018). Notably, in the ischemic brain, OX40L was expressed by cDC2 cells and not by other infiltrating DC populations or microglia. We previously demonstrated deleterious effects of cDC2s mediated by IL-17-driven innate immune responses that exacerbate the brain lesion after stroke (Gelderblom et al., 2018). In the present study DCs, but not CD11c^{-} and CD11c^{+} microglia, obtained from the ischemic brain were able to induce proliferation of CD4^{+} or CD8^{+} T cells. In other

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neurological conditions, MHCI-restricted CD8$^+$ T cell priming is dependent on DCs (Malo et al., 2018). In experimental autoimmune encephalomyelitis (EAE), antigen presentation by peripheral DCs is necessary for reactivation of myelin oligodendrocyte glycoprotein (MOG)-reactive encephalitogenic T cells (Greter et al., 2005), and a population of cDCs samples and presents myelin antigens and is critical for licensing T cells to initiate neuroinflammation (Mundt et al., 2019). Wlodarczyk et al. (2014) specifically addressed the capacity of CD11c$^-$ and CD11c$^+$ microglia to present antigen versus infiltrating CD11c$^+$ cells in EAE. This study showed equivalent ability to induce proliferation of MOG-immunized CD4$^+$ T cells by CNS-resident CD11c$^+$ microglia or blood-derived CD11c$^+$ cells, but the former were unable to induce pathogenic T cell responses. Although it is possible that the disease-specific environment determined the maturation status of CD11c$^+$ microglia and shaped the capacity of these cells to interact with T cells, the different studies seem to agree in that CD11c$^+$ microglia has lower capacity of pathogenic T cell induction than infiltrating DCs.

We identified cDC1 among the infiltrating CD11c$^+$ cells and found expression of *Clec9a*, which may contribute to sensing necrotic cell death and modulating the inflammatory response (del Fresno et al., 2018; Sancho et al., 2009). Infiltration of cDC1 to the ischemic brain was, at least in part, dependent on CCR1, and cDC1s were protective in brain ischemia. cDC1 play critical roles in inducing T helper 1 and cytotoxic T cell immunity because of their capacity to cross-present antigen (Hildner et al., 2008). Moreover, cDC1 regulate complex innate immune responses (Janela et al., 2019; Del Fresno and Sancho, 2019) that may contribute to their effect on stroke outcome. Under pathological conditions, several lines of evidence support the participation of cDC1 in induction of tolerance mediated by promoting inducible Treg cells in several experimental settings (Coombes et al., 2007; Sun et al., 2007; Toubai et al., 2010; Khare et al., 2013; Arnold et al., 2019). This effect may be relevant for stroke outcome because Treg cells play critical immunomodulatory functions in brain ischemia and exert protective effects (Liesz et al., 2009). These results underscore the need to further investigate DC function in brain diseases because DC-based druggable targets might open new therapeutic avenues for neuroinflammatory conditions.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.celrep.2020.108291.

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AUTHOR CONTRIBUTIONS

Conceptualization, A.M.P., F.M.-M., D.S., C.d.F., M. Gelderblom, T.M., and M. Gallizioli; Methodology, F.M.-M., M. Gallizioli, M. Gelderblom, E.C., and C.J.; Formal Analysis, A.M.P., M. Gallizioli, and F.M.-M; Investigation, M. Gallizioli, F.M.-M., A.O.-d.-A., A.S.-P., R.C., C.J., V.H.B., F.R.-J., M.A.-R., J.P., and E.B.-T.; Resources, D.S. and C.d.F.; Writing – Original Draft, A.M.P.; Writing – Review & Editing: M. Gallizioli, A.M.P., F.M.-M., D.S., C.d.F., M. Gelderblom, E.C., and T.M.; Visualization, A.M.P., M. Gallizioli, and F.M.-M.; Supervision, A.M.P. and F.M.-M.; Project Administration, A.M.P.; Funding Acquisition, A.M.P., D.S., F.M.-M., and C.d.F.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-mouse CD11b (clone M1/70, APC-Cy7	BD Biosciences	557657; RRID:AB_396772
Anti-mouse CD11b (clone M1/70, BV605)	BioLegend	101237; RRID:AB_11126744
Anti-mouse CD45 (clone 30-F11, BV786)	BD Biosciences	564225; RRID:AB_2716861
Anti-mouse CD45 (clone 30-F11 APC-eFluor780)	Thermo Fisher Scientific	47-0451-82; RRID:AB_1548781
Anti-mouse Ly6G (clone 1A8, PE-Cy7)	BD Biosciences	560601; RRID:AB_1727562
Anti-mouse Ly6G (clone 1A8, Alexa Fluor 700)	BioLegend	127621; RRID:AB_10640452
Anti-mouse F4/80 (clone BM8, BV605)	BioLegend	123133; RRID:AB_2562305
Anti-mouse Ly6C (clone HK1.4, eFluor 450) or	Thermo Fisher Scientific	48-5932-82; RRID:AB_10805519
Anti-mouse Ly6C (clone HK1.4, PerCP/Cy5.5)	BioLegend	128011; RRID:AB_1659242
Anti-mouse XCR1 (clone ZET, BV421)	BioLegend	148216; RRID:AB_2565230
Anti-mouse CD103 (clone M290, BV711)	BD Biosciences	564320; RRID:AB_2738743
Anti-mouse B220 (clone RA3-6B2, BV785)	BioLegend	103245; RRID:AB_11218795
Anti-mouse MHCII (I-A/I-E, clone M5/114.15.2, FITC)	Thermo Fisher Scientific	11-5321-82; RRID:AB_465232
Anti-mouse MHCII (I-A/I-E,clone M5/114.15.2, BV711)	BioLegend	107643; RRID:AB_2565976
Anti-mouse CD172a (clone P84, PE)	BD Biosciences	560107; RRID:AB_1645248
Anti-mouse CD64 (clone X54-5/7.1, PE-Cy7)	BioLegend	139313; RRID:AB_2563903
Anti-mouse CD11c (clone N418, APC)	Thermo Fisher Scientific	17-0114-82; RRID:AB_469346
Anti-mouse CD11c (clone N418, FITC)	Bio-Rad	MCA1369F; RRID:AB_324141
Anti-mouse CD31 (clone 390, PE-Cy7)	Thermo Fisher Scientific	25-0311-82; RRID:AB_2716949
Anti-mouse CD45 (clone 30-F11, FITC)	BD Biosciences	561088; RRID:AB_10562038
Anti-mouse CD11b (clone M1/70, Alexa Fluor 647)	BD Biosciences	557686; RRID:AB_396796
Anti-Mouse CD16/CD32 (Mouse BD Fc Block, Clone 2.4G2)	BD Biosciences	553142; RRID:AB_394657
Anti-Mouse ACSA2 (Clone IH3-18A3, PE)	Miltenyi Biotec	130-102-365; RRID:AB_2651189
Rabbit polyclonal anti-mouse laminin	Agilent	Z0097; RRID:AB_2313665
Anti-mouse P2RY12	AnaSpec	55043A; RRIB:AB_2298886
Anti-mouse Iba1	Wako Chemicals	016-20001; RRID:AB_839506
Anti-mouse Myelin Basic Protein [BDI221]	Abcam	ab66188; RRID:AB_1141067
Chemicals, Peptides, and Recombinant Proteins		
PLX5622	Plexxikon Inc.	N/A
Recombinant Murine Flt3-Ligand	Peprotech	250-31L
Collagenase from Clostridium histolyticum	Sigma Aldrich	C5138
Deoxyribonuclease I from bovine pancreas	Sigma Aldrich	D5025-150KU
Percoll®	GE Healthcare	17-0891-01
HBSS, no calcium, no magnesium, no phenol red	Thermo Fisher Scientific	14175-053
LIVE/DEAD Fixable Aqua Dead Cell Stain Kit, for 405 nm excitation	Thermo Fisher Scientific	L34957
HBSS, calcium, magnesium, no phenol red	Thermo Fisher Scientific	14025-092
Stain Buffer (FBS)	BD Biosciences	554656
Neural Tissue Dissociation Kit (P)	Miltenyi Biotec	130-092-628
Myelin Removal Beads II	Miltenyi Biotec	130-096-433
DPBS no calcium, no magnesium	Thermo Fisher Scientific	14190-094

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Adult Brain Tissue Dissociation Kit	Miltenyi Biotec	130-107-677
Debris Removal Solution	Miltenyi Biotec	130-109-398
Red Blood Cell Removal Solution	Miltenyi Biotec	130-094-183
Anti-ACSA-2 microbead kit, mouse	Miltenyi Biotec	130-097-678
AstroMACS Separation Buffer	Miltenyi Biotec	130-117-336
Pan T Cell Isolation Kit II	Miltenyi Biotec	130-095-130
CellTrace Violet Cell Proliferation Kit	Invitrogen	C34557
Recombinant murine IL-2	Peprotech	212-12
Ovalbumin (323-339) (chicken, Japanese quail)	Sigma Aldrich	O1641
Ovalbumin (257-264) (chicken) SIINFEKL	Sigma Aldrich	S7951
Recombinant Murine IL-4	Peprotech	214-14
Lipopolysaccharides from *Escherichia coli* O55:B5	Sigma Aldrich	L2880
TO-PRO-3 Iodide (642/661)	Thermo Fisher Scientific	T3605
High Capacity cDNA Reverse Transcription kit	Applied Biosystems	4387406
TaqMan® Pre Amp Master Mix (2 ×)	Thermo Fisher Scientific	4384266
TE Buffer, Tris-EDTA, 1X Solution, pH 8.0	Fisher Scientific	BP2473
TaqMan Universal PCR Master Mix	Thermo Fisher Scientific	4304437
TruSeq Stranded mRNA Sample Prep Kit v2	Illumina	RS-122-2101/2
SuperScript II reverse transcriptase	Invitrogen	18064-014
SMART-Seq v4 Ultra Low Input RNA kit	Clontech	634890
NEBNext Ultra DNA Library Prep kit	New England Biolabs	E7370
DiI (1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate)	Sigma Aldrich	42364
Critical Commercial Assays		
KAPA Library Quantification Kit	KapaBiosystems	KK4835
Deposited Data		
RNA-Seq data	This paper	GEO: GSE136856
Experimental Models: Organisms/Strains		
Mouse: CD11c-eYFP; Tg(Itgax-Venus)1Mnz	The Jackson Laboratory	SN8829
Mouse: CX3CR1Cre^{ERT2}; B6.129P2(C)-Cx3cr1tm2.1 (cre/ERT2)Jung/J	The Jackson Laboratory	SN020940
Mouse: ROSA26-tdTomato; (B6.Cg-Gt(ROSA) 26Sortm9(CAG-tdTomato)Hze/J	The Jackson Laboratory	SN007909
Mouse: Rag1/OTI; B6.129S7-Rag1tm1Mom Tg(TcraTcrb)1100Mjb	Taconic	4175
Mouse: OTII; B6.Cg-Tg(TcraTcrb)425Cbn/J	The Jackson Laboratory	SN004194
Mouse: C57BL/6J	Janvier	SC-C57J-M
Oligonucleotides		
See Table S4		N/A
Software and Algorithms		
R software, DESEQ2 package	(Love, Huber, and Anders 2014)	https://bioconductor.org/packages/release/bioc/html/DESeq2.html
FacsDiva v5	BD Biosciences	https://www.bdbiosciences.com/en-us/instruments/research-instruments/research-software/flow-cytometry-acquisition/facsdiva-software
FlowJo v10	FlowJo LLC	https://www.flowjo.com/solutions/flowjo
FastQC v0.11.5	Babraham Bioinformatics	https://www.bioinformatics.babraham.ac.uk/projects/download.html#fastqc
Skewer (v0.2.2)	(Jiang et al., 2014)	http://bioweb.pasteur.fr/packages/pack@skewer@0.2.2

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Immgen 'Population Comparison tool'	(ImmGen Consortium 2016)	http://rstats.immgen.org/PopulationComparison/
Immgen 'My Gene Set tool'	(ImmGen Consortium 2016)	http://rstats.immgen.org/MyGeneSet_New/index.html
DAVID Bioinformatics Resources 6.7	(Huang, Sherman, and Lempicki 2009)	https://david.ncifcrf.gov/home.jsp
Cytoscape v3.5.1	(Shannon et al., 2003)	http://chianti.ucsd.edu/cytoscape-3.5.1/
Prism v.8	GraphPad	https://www.graphpad.com/scientific-software/prism/

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Anna M. Planas (anna.planas@iibb.csic.es).

Materials Availability

This study did not generate new unique reagents.

Data and Code Availability

The RNA-Seq data are accessible from the GEO repository of the National Center for Biotechnology Information, U.S. National Library of Medicine (The accession number for these data is GEO: GSE136856) (https://www.ncbi.nlm.nih.gov/geo/info/linking.html). Other datasets are available from the corresponding author upon reasonable request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animals

We used adult (3-4 month old) mice in the C57BL/6 background. We used CD11c-eYFP reporter mice (Tg(Itgax-Venus) 1Mnz; #SN8829) that were maintained as hemizygous by crossing with C57BL/6 mice; CX3CR1cre^{ERT2} mice (B6.129P2(C)-Cx3cr1tm2.1(cre/ERT2)Jung/J, #SN020940) crossed with reporter ROSA26-tdTomato mice (B6.Cg-Gt(ROSA) 26Sortm9(CAG-tdTomato)Hze/J, #SN007909) that received tamoxifen three weeks before surgery; and OTII mice (B6.Cg-Tg(TcraTcrb)425Cbn/J, #SN004194); all obtained from The Jackson Laboratory. Colonies of *Batf3*$^{-/-}$ mice and *Batf3*$^{+/+}$ wild-type mice (Enamorado et al., 2017) and OTI mice (OT-I transgenic mice – C57BL/6-Tg(TcraTcrb)11003Mjb/J, #003831 – crossed with Rag1$^{-/-}$ mice – B6.129S7-Rag1tm1Mom/J, #002216 – both from The Jackson Laboratories) were kept under SPF in the animal house of CNIC and were transferred to the animal house of the School of Medicine of UB for induction of ischemia. Additional C57BL/6 wild-type mice (#SC-C57J-M) were purchased from Janvier (France). Prior to experiments all mice were maintained under SPF conditions in the animal house of the School of Medicine of the University of Barcelona (UB). Unless otherwise stated we used male mice. Studies of parabiosis were carried out in female mice and corresponding non-parabiotic female mice were used for comparative purposes. We always compared mice of the same sex given that we did not investigate sex effects in this study. Animal work was conducted following the Catalan and Spanish laws (Real Decreto 53/2013) and the European Directives. All experiments were conducted with approval of the ethical committee (Comité Ètic d'Experimentació Animal, CEEA) of UB, and the local regulatory bodies of the Generalitat de Catalunya, and in compliance with the NIH Guide for the Care and Use of Laboratory Animals. The work is reported following the ARRIVE guidelines.

Primary cell cultures

We prepared primary glial cell cultures from the cerebral cortex of 1-to 2-day-old newborn C57BL/6 mice of both sexes as reported (Bonfill-Teixidor et al., 2017). In brief, we removed the meninges and dissociated the tissue with 0.025% trypsin containing 1mM EDTA (GIBCO) under shaking for 30 min at 37°C. We then added DMEM:F12 with 10% FBS (GIBCO) and 4mg/mL DNase (Sigma-Aldrich). After centrifugation at 300 x*g* for 5 min, cells were seeded in DMEM-F12 with 20% FBS and 1% penicillin/streptomycin (P/S) 10,000 U/10,000 μg/mL (GIBCO) at a density of 250,000 cells/mL in 25cm^2 tissue culture flasks. The cells were maintained at 37°C in a humidified atmosphere of 5% CO_2-95% air for 8 days. Cells were subcultured at 8 days *in vitro*. After two washes in PBS, cells were treated with 0.125% trypsin-EDTA for 5 min at 37°C. Neutralization with supplemented media (1:1) was carried out and cells were seeded at 80,000 cells/mL in DMEM-F12 with 20% FBS and 1% P/S in poly-D-lysine-coated tissue culture plates. At confluence, cells were treated with mouse recombinant IL-4 (50 ng/mL, #214-14, Peprotech) or LPS (10 ng/mL; *Escherichia coli* 055:B5,# L2880 Sigma-Aldrich) and the cells were obtained at different time points for RNA extraction.

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METHOD DETAILS

Study design

Our main objective was to compare microglia versus dendritic cells in different experimental groups. Randomization and blinding was conducted for treatment administration when possible, as stated below. Cerebral blood flow (CBF) data were used as predefined criteria to exclude animals from the study.

Induction of brain ischemia

Cerebral ischemia was induced by transient (45-min) occlusion of the right middle cerebral artery (MCAo) with the intraluminal technique, as described (Salas-Perdomo et al., 2018). In brief, anesthesia was induced with 4% isoflurane in a mixture of 30% O_2 and 70% N_2O and it was maintained with 1%–1.5% isoflurane in the same mixture by the aid of a facial mask. A longitudinal cut was produced in the ventral middle line of the neck to expose and ligate the right common carotid artery (CCA). Next the submaxillary glands and the omohyoid and sternothyroid muscles were separated, exposing the carotid arteries. A monofilament (#701912PK5Re, Doccol Corporation, Sharon, MA) was introduced through the right external carotid artery up to the level where the MCA branches out. CBF was monitored with laser Doppler flowmetry (Perimed, AB, Järfälla, Sweden). After 45 min of arterial occlusion, the filament was cautiously removed, and the suture of the ipsilateral CCA was removed to allow reperfusion. Mice received analgesia (buprenorphine, 150 μl of a 0.015 mg/mL solution, via i.p.) and were kept on a thermal blanket for 1 hour after surgery. Cerebral blood flow (CBF) data were used as predefined criteria to exclude animals from the study as follows: (1) mice with incorrect surgery or surgical complication; (2) mice that did not show a drop in CBF greater than 75% from basal CBF values after introducing the filament in the middle cerebral artery , as it was considered that ischemia was not successfully induced; and (3) mice that did not show recovery in CBF of higher than 70% of the basal value as reperfusion did not reach an adequate level.

Drug treatments

For microglia depletion, mice received the CSF1R inhibitor PLX5622 (Plexxikon Inc, Berkeley USA) following previously reported protocols (Otxoa-de-Amezaga et al., 2019). The inhibitor was mixed into AIN-76A standard chow at 1200 ppm (Brogaarden, Denmark). Mice (8-week old) received the diet *ad libitum* for three weeks prior to induction of ischemia and the diet was maintained until the mice were killed.

Treatment controls received AIN-76A chow for the same period of time. Both diets were given in parallel in groups of 5 animals per cage. Researchers conducting ischemia and obtaining further data were not aware of the identity of the diet groups.

Flt3L (10 μg per mouse, #250-31L, Peprotech) was administered during 7 days using Alzet pumps (1007D, #0000290, Durect Corporation, USA) implanted subcutaneously. Ischemia was performed 3 days after pump implantation and mice were euthanized 4 days post-ischemia.

Daily injections of CCR1 antagonist J113863 (Tocris #2595, 10 mg/Kg dose, 300 μl/injection) were given i.p. starting 1 day post-ischemia and ending at day 3. Treatment controls received the same volume of the vehicle (phosphate buffer containing 10% Tween80). The dose of J113863 was decided following previous studies (Amat et al., 2006). Treatment was randomly assigned and was administered in a blinded fashion. The animals received a code that did not reveal the identity of the groups.

Induction of Parabiosis

We generated parabiotic pairs by joining 3-month old female CD11c-eYFP mice with wild-type female littermates, as reported (Torres et al., 2015). We obtained the brain tissue three weeks later. In some parabiotic pairs we induced ischemia in the wild-type mouse and the brain was obtained 4 days post-ischemia. For comparison purposes, we induced ischemia in non-parabiotic CD11c-eYFP and CX3CR1cre^{ert2}-ROSA26 tdT female mice.

Generation of chimeric mice

We generated chimeric mice by chemical ablation of the bone marrow of WT recipient mice followed by transplantation of bone marrow from either WT or *Batf3*$^{-/-}$ donor mice, as described (Kierdorf et al., 2013). In brief, adult (2-month old) male WT mice received three intraperitoneal injections of the chemotherapeutic agent busulfan (30 μg/g body weight) 7, 5 and 3 days prior to the transfer of five million bone marrow cells from Baft3$^{-/-}$ or WT donor mice via the tail vein. Ischemia was induced eight weeks after grafting.

Brain tissue processing and Flow cytometry

Mice were anesthetized and perfused transcardially with 30 mL PBS containing heparin (5 U/mL). The ischemic cortex (ipsilateral) and the corresponding region of the non-affected hemisphere (contralateral) were dissected and analyzed separately. The tissue was homogenized with gentleMACS Dissociator (#130-096-427, Miltenyi Biotec: 2x Brain_1 program and 1x ABDK_37C program by Miltenyi) while immersed in 2ml of HBSS buffer (w/o Ca^{2+} and Mg2+, #14175-053, Thermo Fisher Scientific) containing 100 U/mL collagenase IV (#C5138, Sigma) and 50 U/mL DNase I (#D5025-150 KU, Sigma). The tissue was then filtered on a cell strainer (70um) and the cells were separated from myelin and debris with a 30% percoll gradient (#17-0891-01, GE Healthcare) in HBSS without Ca^{2+} and Mg2+ centrifuged at 950 g during 20min without brakes. Cells were collected from the bottom of the

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tube after centrifugation and washed with FACS buffer (PBS, 2 mM EDTA, 2% FBS). Unspecific binding of antibodies was blocked by previous incubation for 10 min with anti CD16/CD32 (Fc block, clone 2.4G2, BD PharMingen) in FACS buffer at 4°C. Live/dead Aqua cell stain (Thermo Fisher Scientific #L34957) was used to determine the viability of cells. Cells were incubated with the following primary antibodies during 20 minutes at 4°C: CD11b (clone M1/70, APC-Cy7, BD PharMingen #557657 or clone M1/70, Brilliant Violet 605, Biolegend #101237), CD45 (clone 30-F11, Brilliant Violet 786,BD Horizon #564225 or clone 30-F11 APC-eFluor780, eBioscience #47-0451-82), Ly6G (clone 1A8, PE-Cy7, BD Biosciences #560601 or clone 1A8, Alexa Fluor 700, Biolegend #127621), Ly6C (clone HK1.4, eFluor 450, eBioScience #48-5932-82 or clone HK1.4, PerCP/Cy5.5, Biolegend #128011), XCR1 (clone ZET, Brilliant Violet 421, Biolegend #148216), CD103 (clone M290, Brilliant Violet 711, BD Biosciences #564320), B220 (clone RA3-6B2, Brilliant Violet 785, Biolegend #103245), MHCII (clone M5/114.15.2, FITC, eBioscience #11-5321-82 or clone M5/114.15.2, Brilliant Violet 711, Biolegend #107643), CD172a (clone P84, PE, BD Biosciences #560107), CD64 (clone X54-5/7.1, PE-Cy7, Biolegend #139313), CD11c (clone N418, APC, eBioscience #17-0114-82 or clone N418, FITC, Bio-Rad #MCA1369F). Data was acquired in a BD LSRII cytometer using FacsDiva software (version 5, BD Biosciences, San Jose, CA, USA). Flow-Count Fluorospheres (Beckman-Coulter # 7547053) were used for absolute quantification. Data analyses were performed with FlowJo software (version 10, FlowJo LLC, Ashland, OR, USA).

Cell isolation

Myeloid cells, astrocytes, and endothelial cells were isolated from the brain of adult mice (13-14 weeks old) using different animals and procedures for each cell type. CD11c-eYFP^{+} cells and microglia were isolated from the brain of CD11c-eYFP mice or CX3CR1cre^{ERT2}-ROSA26 tdT mice respectively using Fluorescence Activated Cell Sorting (FACS). Brains were collected in cold HBSS buffer (w/o Ca^{2+} and Mg^{2+}, #14175-053, Thermo Fisher Scientific). The brain tissue was enzymatically dissociated using the Neural Tissue Dissociation Kit (P) (#130-092-628, Miltenyi Biotec). The gentleMACS Dissociator (#130-096-427, Miltenyi Biotec) was used for mechanical dissociation steps following the Neural Tissue Dissociation Kit (P) protocol for dissociation without heaters. The digested tissue was filtered twice with 70 μm and 40 μm filters washing with HBSS (with Ca^{2+} and Mg^{2+}, #14025-092, Thermo Fisher Scientific). Cells were separated from myelin and debris by 30% isotonic percoll gradient (#17-0891-01, GE Healthcare) in Myelin Gradient Buffer (MGB). Samples were centrifuged at 950 xg for 30 min without acceleration or brake. Cells were collected from the bottom of the tube, washed once with FACS Stain Buffer (#554656, BD Biosciences), and processed for FACS (no staining required) in a FACSAriaII sorter (BD Biosciences).

Endothelial cells were isolated from the brain of adult wild-type mice by FACS. Brains were collected in cold HBSS buffer (w/o Ca^{2+} and Mg^{2+}). The brain tissue was enzymatically dissociated using the Neural Tissue Dissociation Kit (P) (#130-092-628, Miltenyi Biotec) and mechanical dissociation was carried out with the gentleMACS Dissociator. The digested tissue was filtered in 70 μm filters washing with HBSS (with Ca^{2+} and Mg^{2+}). Cells were separated from myelin by magnetic separation using Myelin Removal Beads II (#130-096-433, Miltenyi Biotec) and LS Columns according to manufacturer's instructions. Cells were resuspended in FACS buffer (PBS, 2 mM EDTA, 2% FBS). Fc receptors were blocked by previous incubation for 10 min with CD16/CD32 (clone 2.4G2, BD PharMingen) in FACS buffer at 4°C. Live/dead Aqua cell stain (#L34957, Invitrogen) was used to determine cell viability. Cells were incubated with the following primary antibodies: CD31 (# 25-0311-82, clone 390, PE-Cy7, BD Biosciences), CD45 (#561088, clone 30-F11, FITC, BD Biosciences), CD11b (#557686, clone M1/70, Alexa Fluor 647, BD Biosciences), and cells processed for FACS in a FACSAriaII sorter (BD Biosciences).

Astrocytes were isolated from the brain of adult wild-type mice (13-14 weeks old) using immunomagnetic separation (Miltenyi Biotec, Germany). Brains were collected in cold D-PBS buffer (#14190-094, Thermo Fisher Scientific). The brain tissue was enzymatically dissociated using the Adult Brain Tissue Dissociation Kit (#130-107-677, Miltenyi Biotec). The gentleMACS Dissociator was used for mechanical dissociation steps during 30 min at 37°C (program 37C_ABDK_01). The digested tissue was filtered (70 μm) with D-PBS. Myelin and cell debris are removed using the Debris Removal Solution (#130-109-398, Miltenyi Biotec) followed by removal of erythrocytes using the Red Blood Cell Removal Solution (#130-094-183, Miltenyi Biotec). Then, cells were magnetically labeled with Anti-ACSA-2 microbeads (#130-097-678, Miltenyi Biotec) diluted in AstroMACS Separation Buffer (#130-117-336, Miltenyi Biotec) for 15 min in the dark in the refrigerator (2–8°C). ACSA-2+ cells were collected using magnetic field columns. Cell purity was assessed by using the Anti-ACSA-2-PE clone IH3-18A3 (#130-102-365, Miltenyi Biotec).

Antigen-specific T cell proliferation assay

CD11c-eYFP^{+} cells and microglia were obtained from the ischemic brain tissue (day 4) and isolated as described above. Cells were resuspended in FACS buffer (PBS, 2 mM EDTA, 2% FBS). Fc receptors were blocked by previous incubation for 10 min with CD16/CD32 (clone 2.4G2, BD PharMingen) in FACS buffer at 4°C. Cells were incubated with the following primary antibodies: CD45 (clone 30-F11 APC-eFluor780, eBioscience #47-0451-82) and CD11b (#557686, clone M1/70, Alexa Fluor 647, BD Biosciences) and processed for FACS in a FACSAriaII sorter (BD Biosciences). For studies of class II antigen presentation, T cells were isolated from spleen of OTII mice by magnetic separation using Pan T Cell Isolation Kit II (#130-095-130, Miltenyi Biotec) according to manufacturer's instructions for manual cell separation with LS columns. T cells were stained with CellTrace Violet Cell Proliferation Kit (2 μM, #C34557, Invitrogen). Cells were co-cultured for 5 days at a 1:10 APC to T cell ratio in RPMI 1640 complete medium in the presence of recombinant murine IL-2 (10 ng/mL, #212-12, Peprotech) and OVA peptide 323-339 (2 μg/mL, #O1641, Sigma). The cells were washed with FACS buffer and data was acquired in a BD LSRII cytometer using FacsDiva software (version 5, BD Biosciences,

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San Jose, CA, USA). The proliferation index is expressed as % of cells with reduction of the intensity of CellTrace Violet Staining. For studies of class I antigen presentation, CD8 T cells were isolated from the spleen of Rag1KO-OTI mice and stained with CellTrace Violet Cell Proliferation Kit as above. APCs were pulsed during 1h with SIINFEKL peptide (0.1 ng/mL, #S7951, Sigma) in RPMI 1640 complete medium and then extensively washed with RPMI 1640 complete medium. T Cells and peptide-pulsed APCs were co-cultured for 3 days at a 1:6 APC to T cell ratio in RPMI 1640 complete medium. After washing with FACS buffer, data was acquired in a BD LSRII cytometer using FacsDiva software (version 5, BD Biosciences, San Jose, CA, USA). The proliferation index is expressed as % of cells with reduction of the intensity of CellTrace Violet Staining.

Immunofluorescence

Mice were perfused through the heart with 40 mL of cold saline solution (0.9% NaCl) followed by 20 mL of cold 4% paraformaldehyde diluted in phosphate buffer (PB) pH 7.4. The brain was removed, fixed overnight with the same fixative, immersed in 30% sucrose in PB for cryoprotection for at least 48 h, and frozen in isopentane at −40°C. Cryostat brain sections (14-μm thick) were fixed in ethanol 70%, blocked with 3% normal serum, and incubated overnight at 4°C with primary antibodies: rabbit polyclonal antibodies against pan-laminin (1:100, #Z0097, Dako), P2RY12 (1:250, #AS-55043A, AnaSpec Inc.), or ionized calcium-binding adaptor molecule-1 (Iba-1) (1:100, #016-20001, Wako Chemicals), or mouse monoclonal antibody against myelin basic protein (1:200, BDI221, #ab66188, Abcam) followed by secondary antibodies (Alexa Fluor®; Thermo Fisher Scientific). Cell nuclei were stained with To-Pro3 (#T3605, Thermo Fisher Scientific). Images were obtained in a confocal microscope (TCS SPE-II, Leica Microsystems) with LAS software (Leica). Images were no further processed. Figures were prepared with Adobe Photoshop. When required for illustrative purposes contrast was enhanced globally in the whole image.

RNA Extraction

RNA was extracted from samples of FACS-sorted CD11c-eYFP$^+$ cells, microglia, astrocytes and endothelial cells with PureLink RNA Micro Kit (#12183016, Invitrogen) following manufacturer indications with minor modifications. RNA was precipitated with 70% ethanol overnight at −20°C. To avoid genomic DNA contamination a DNase step was performed. RNA quantity and purity were assessed with the High Sensitivity RNA ScreenTape® (Agilent 2200 TapeStation system). We also extracted RNA from brain tissue using Trizol® Reagent (Life Technologies) followed by the PureLink RNA Mini Kit (#12183018A, Invitrogen). In this case, we assessed the RNA quantity and quality using a ND-1000 micro-spectrophotometer (NanoDrop Technologies).

RT-PCR

Total RNA was reverse-transcribed using a mixture of random primers (High Capacity cDNA Reverse Transcription kit, Applied Biosystems, Foster City, CA, USA, #4387406). For RNA obtained from brain tissue, 1000 ng of total RNA were reverse-transcribed and the final product was diluted 6 times in RNase-free water. For samples of FACS-sorted cells, cDNA was pre-amplified following the manufacturer indications (TaqMan® Pre Amp Master Mix (2 ×), #4384266, Thermo Fisher Scientific) in order to have enough cDNA for the real-time-PCR. The pre-amplification step was carried out using a pool of TaqMan probes of interest and the final pre-amplified product was diluted 20 times with tris-EDTA buffer pH 8.0 (#BP2473, Fisher Bioreagents).

Real-time quantitative RT-PCR analysis was carried out with Taqman system (#4304437, Thermo Fisher Scientific) using the iCycler iQTM Multicolor Real-Time Detection System (Bio-Rad). The primers are listed in Table S4 and the qPCR conditions were 2 min at 50°C, 10 min at 95°C followed by 40 cycles of 15 s at 95°C and finally 1 min at 60°C. Quantification was performed by normalizing cycle threshold (Ct) values with the housekeeping gene (Hprt1) Ct, and analysis was carried out with the $2-\Delta\Delta CT$ method.

Transcriptomic analysis

RNA samples were obtained from sorted eYFP$^+$ cells of the ipsilateral brain hemisphere of CD11c-eYFP mice 4 days post-ischemia, the spleen of control CD11c-eYFP mice and the ipsilateral brain hemisphere of the WT mice of parabiotic CD11c-eYFP/WT pairs, and from microglial cells sorted from control brain and the ipsilateral brain hemisphere 4 days post-ischemia of Cx3cr1Cre^{ERT2}:ROSA26-tdT mice. Samples had RIN values > 8.

For the first RNA-Seq comparison (i.e., CD11c-eYFP+ cells of ischemic brain, CD11c-eYFP$^+$ spleen cells, CX3CR1$^+$ microglia of control and ischemic brain), libraries were prepared using the TruSeq Stranded mRNA Sample Prep Kit v2 (#RS-122-2101/2, Illumina) according to the manufacturer's protocol. Briefly, from 2.4 to 147 ng of total RNA were used for poly(A)-mRNA selection using streptavidin-coated magnetic beads and were subsequently fragmented to approximately 300bp. cDNA was synthesized using SuperScript II reverse transcriptase (#18064-014, Invitrogen) and random primers. The second strand of the cDNA incorporated dUTP in place of dTTP. Double-stranded DNA was further used for library preparation. dsDNA was subjected to A-tailing and ligation of the barcoded Truseq adapters. All purification steps were performed with AMPure XP beads and eluted in 20 μl EB. Library amplification was performed by PCR on selected fragments using the primer cocktail supplied in the kit. Final libraries were analyzed using Agilent DNA 1000 chip to estimate the quantity and check size distribution, and were then quantified by qPCR using the KAPA Library Quantification Kit (#KK4835, KapaBiosystems) prior to amplification with Illumina's cBot. Libraries were sequenced Single Reads, 50nts on the Illumina HiSeq 2500.

For the second RNA-Seq comparison (i.e., CD11c-eYFP$^+$ cells from ischemic brain, Parabiotic CD11c-eYFP+ cells from the ischemic brain of WT mice, CX3CR1$^+$ microglia from ischemic brain), libraries were prepared using the SMART-Seq v4 Ultra Low

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Input RNA kit (#634890, Clontech) and NEBNext Ultra DNA Library Prep kit (#E7370, New England) according to the manufacturer's protocol. Briefly, 1 or 5 ng of total RNA were converted to cDNA using the SMART-seq kit. Long distance PCR was performed for 9 (5 ng) or 11 (1 ng) cycles. Agilent 2100 Bioanalyzer (High Sensitivity DNA Chip) was used for quantification and validation of the obtained cDNA. Ten ng of the resultant cDNA was fragmented using the Covaris S2 instrument in a final volume of 55 μL and adjusting the settings as follows: 10% duty cycle, intensity 5, and 200 cycles per burst for 5 minutes. The resultant fragments were used for the library synthesis using the NEBNext Ultra kit. Briefly, fragments were subjected to end repair and addition of "A" bases to 3′ ends, ligation of adapters and USER excision. Library amplification was performed through 10 cycles of PCR using different indexed primers for multiplexing. All purification steps were performed using AgenCourt AMPure XP beads. Final libraries were analyzed using Agilent DNA High Sensitivity chip to estimate the quantity and to check the size distribution and were then quantified by qPCR using the KAPA Library Quantification Kit (#KK4835, KapaBiosystems). Sequencing was carried out using the Illumina HiSeq 2500, Single Reads, 50nts.

Raw reads were analyzed for data quality using FastQC v0.11.5 (Babraham Bioinformatics) and eventually filtered using skewer v0.2.2 (Jiang et al., 2014) for removing the low quality reads and trimming the Illumina adaptor if necessary. Reads were then aligned to the reference genome (GRCm38) using the annotation from Ensembl (version 88) with STAR aligner (v2.5.3a) using the option "quantMode GeneCounts" for retrieving the gene counts. Differential gene expression analysis was performed using DESEQ2 package (Love et al., 2014), while plots were made using R programming language. We considered as differentially expressed genes that showed a logFC > |1.5| and adjusted p value < 0.001.

ImmGen transcriptomic data comparison

We compared our transcriptomic data with defined ImmGen populations (https://www.immgen.org/) (Miller et al., 2012) following a methodology used in previous studies (Poczobutt et al., 2016), with modifications. The analysis was carried out by Anaxomics Biotech (Barcelona, Spain). In a nutshell, pairwise comparisons between 8 selected ImmGen populations were performed using the Population Comparison tool (http://rstats.immgen.org/PopulationComparison/). The 100 most differential genes in each pairwise comparison were retrieved and a single list of differential genes was generated. We examined the expression of these genes in the comparison between: (a) control brain microglia and control spleen CD11c-eYFP$^+$ cells, (b) microglia versus brain CD11c-eYFP$^+$ cells of ischemic mice, and (c) microglia versus parabiotic brain CD11c-eYFP$^+$ cells of ischemic mice. Genes without a valid value were removed. The expression values of the final lists of genes in each sample (transformed to a Z-score) were used to construct a heatmap representation and an unsupervised gene clustering analysis was performed. The latter analysis was then used for the identification of specific clusters that displayed a differential expression between the studied cohorts. The genes inside these clusters were retrieved and submitted to ImmGen's My Gene Set tool (http://rstats.immgen.org/MyGeneSet_New/index.html). Boxplot representations of the expression value (Log2 of the gene expression value/average expression value of all genes) per each analyzed population were retrieved.

Enrichment analysis

Functional analyses of RNA-Seq data were carried out through the use of DAVID Bioinformatics Resources 6.7 (Huang et al., 2009). Then a hypergeometric enrichment analysis was performed by Anaxomics Biotech to determine the presence of enriched pathways and protein sets in our different cell populations, following previously described methodology (Subramanian et al., 2005). We mapped from mouse genes annotated in the various comparisons of gene expression profiles above described to mouse protein entries according to UniProtKB (The UniProt Consortium, 2017). Genes included followed the criteria: p value < 0.001 and |Log2FC| > 2. The differential genes/proteins were submitted to hypergeometric enrichment analysis (one for each cell population and comparison) to identify the pathways and molecular processes more enriched in each of the cell cohorts (Rivals et al., 2007). Enrichment was run over the following databases: GO terms (Biological Process, Cellular Component, Molecular Function) (Ashburner et al., 2000), KEGG pathways (Kanehisa et al., 2017), pathological conditions and motives included in BED database (Jorba et al., 2020), PharmGKB pathways (Whirl-Carrillo et al., 2012), the pathways from Small Molecule Pathway Database (SMPDB) (Frolkis et al., 2010), and the regulatory molecular mechanisms included in TRRUST database. Cytoscape software v3.5.1 was used to create the representation of individual enriched protein sets based on their enrichment score (Shannon et al., 2003).

Quantification of middle cerebral artery anastomoses

A solution of the lipophilic carbocyanide dye, DiI (DiIC_{18}(3)) was prepared according to a reported protocol (Li et al., 2008). The animals were perfused with 20ml of PBS containing heparin (5 U/mL), 10mL of DiI solution and 10mL of PFA 4% diluted in phosphate buffer consecutively, all at a 5ml/min rate. The brain was removed from the skull, the cortex was dissected out and set on a glass slide for observation. Pictures were taken with an Olympus inverted visible light microscope at 20x and merged to reconstruct the whole cortex surface. Two independent investigators blinded to the genotype of the mice manually counted the number of anastomoses between the middle and the anterior and posterior cerebral arteries. The correlation between the two independent measurements was studied.

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QUANTIFICATION AND STATISTICAL ANALYSIS

Two-group comparisons were carried out with two-tailed Mann–Whitney test or t test, as required after testing for normality, and adjusting for paired measures using Wilcoxon-matched-pairs signed rank test or paired t test. One-sample Wilcoxon test was used to compare with reference values. Multiple comparisons t test and Holm-Sidak method was used. Multiple groups were compared with one-way ANOVA or Kruskal-Wallis test, or two-way ANOVA, followed by appropriate post hoc analyses. In experiments designed to study differences between groups receiving drug treatment, sample size was calculated using G*power 3.1 software (University of Düsseldorf) with an alpha level of 0.05 and a statistical power of 0.8. The size effect (d = 1.1) was estimated based on information on the group mean and SD from previous flow cytometry data of our own laboratory. In experiments designed to study differences in infarct volume between genotypes, the size effect was estimated at 0.97 for a change of 30% based on prior information on infarct volume mean and SD for wild-type mice subjected to ischemia by the same researcher. We built from these numbers the number of animals needed for comparing other outcome measures. For measurements designed as proof of concept, validation, or as internal controls we used minimum reasonable numbers of animals for confirmatory purposes. The specific tests used in each experiment, p values, and n values are stated in Figure Legends. We used GraphPad Prism software version 8.2.0.

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Supplemental Information

Dendritic Cells and Microglia Have Non-redundant Functions in the Inflamed Brain with Protective Effects of Type 1 cDCs

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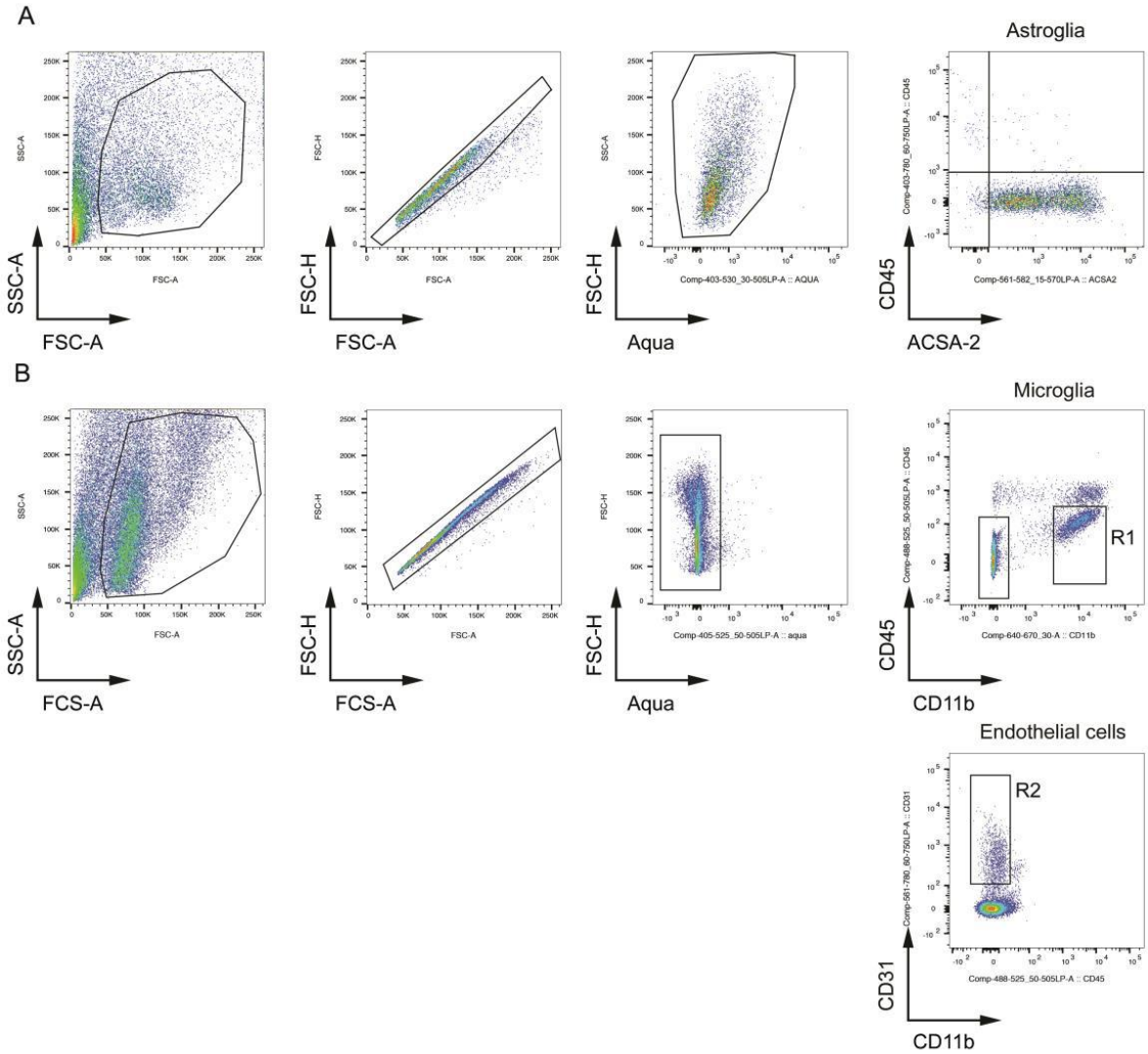
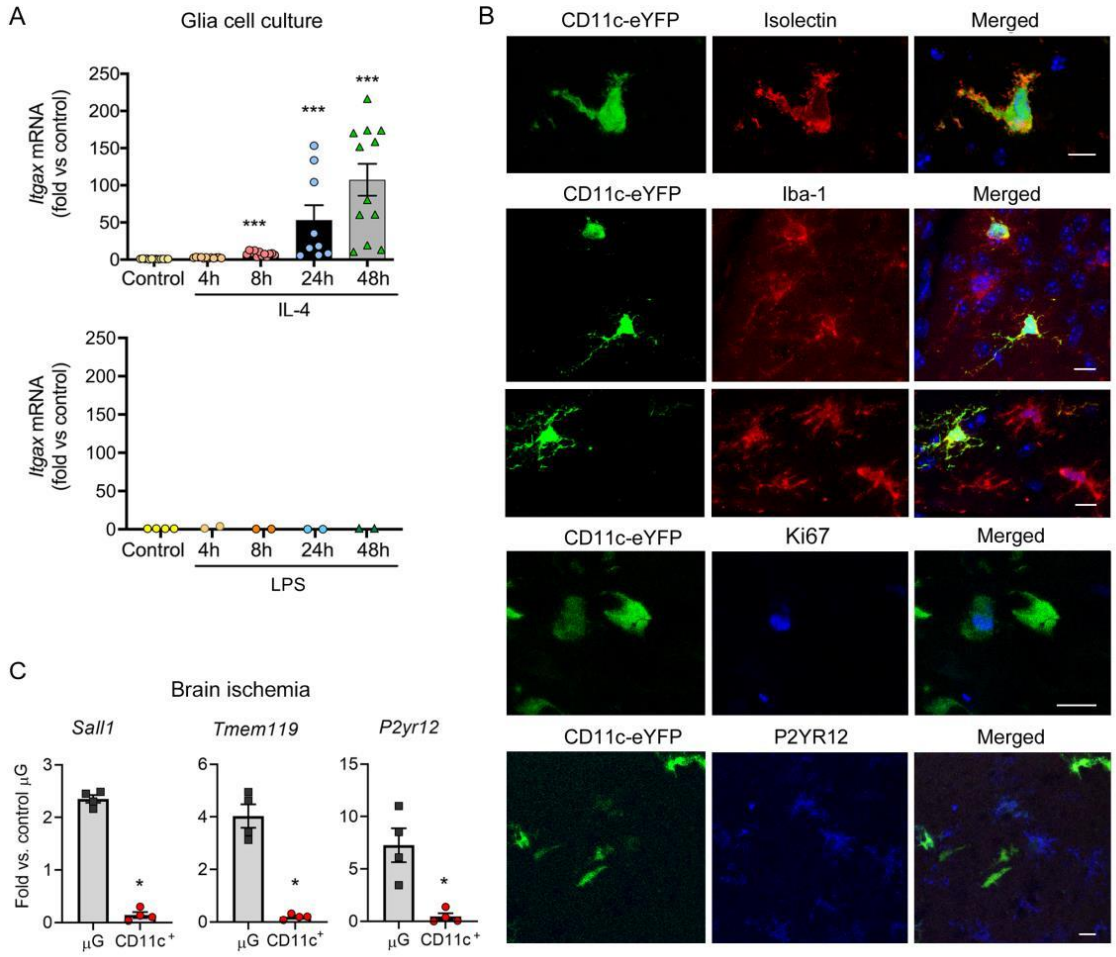
Supplementary Fig S1.



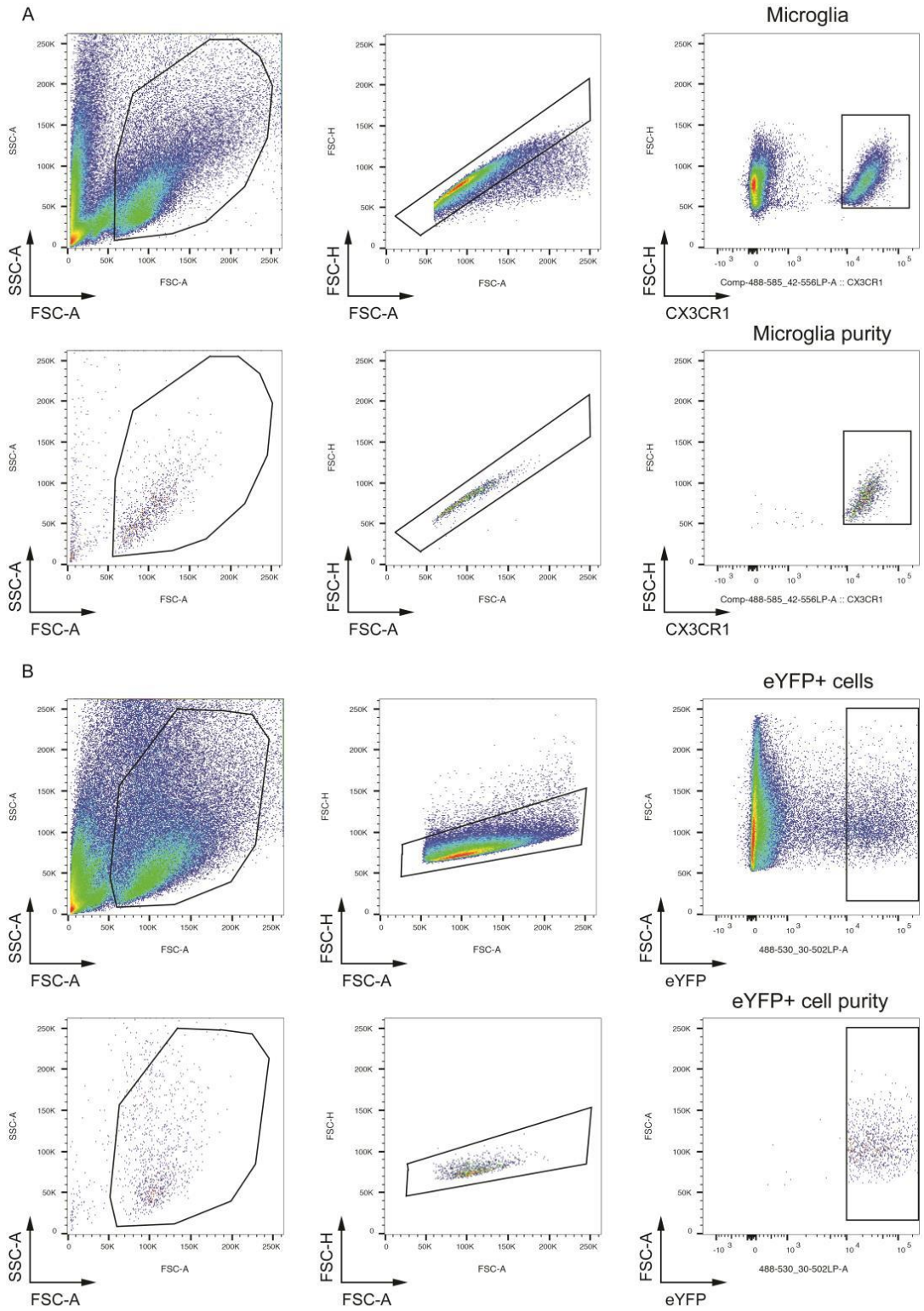
Sup. Fig. S1. Isolation of astroglia, microglia, and endothelial cells from control and ischemic brains. ***Related to Fig. 1F.*** A) Astrocytes were isolated using immunomagnetic separation. We checked the purity by flow cytometry (mean±SD: 92.3±2.7%, n=3) as illustrated in the plots. B) Gating strategy for isolation of microglia and endothelial cells by FACS. Cells were labeled with Aqua Live/Dead staining and CD45, CD11b and CD31 antibodies. After excluding aggregated cells and dead cells, for the microglia gate (R1) we sorted $CD45^{lo}CD11b^{lo}$ cells and for the endothelial cells gate (R2) we sorted $CD45^{-}CD11b^{-}CD31^{+}$ cells. We checked cell purity (n=1 per group): 97.5% for endothelial cells and 92.9% for microglia.

Supplementary Fig. S2



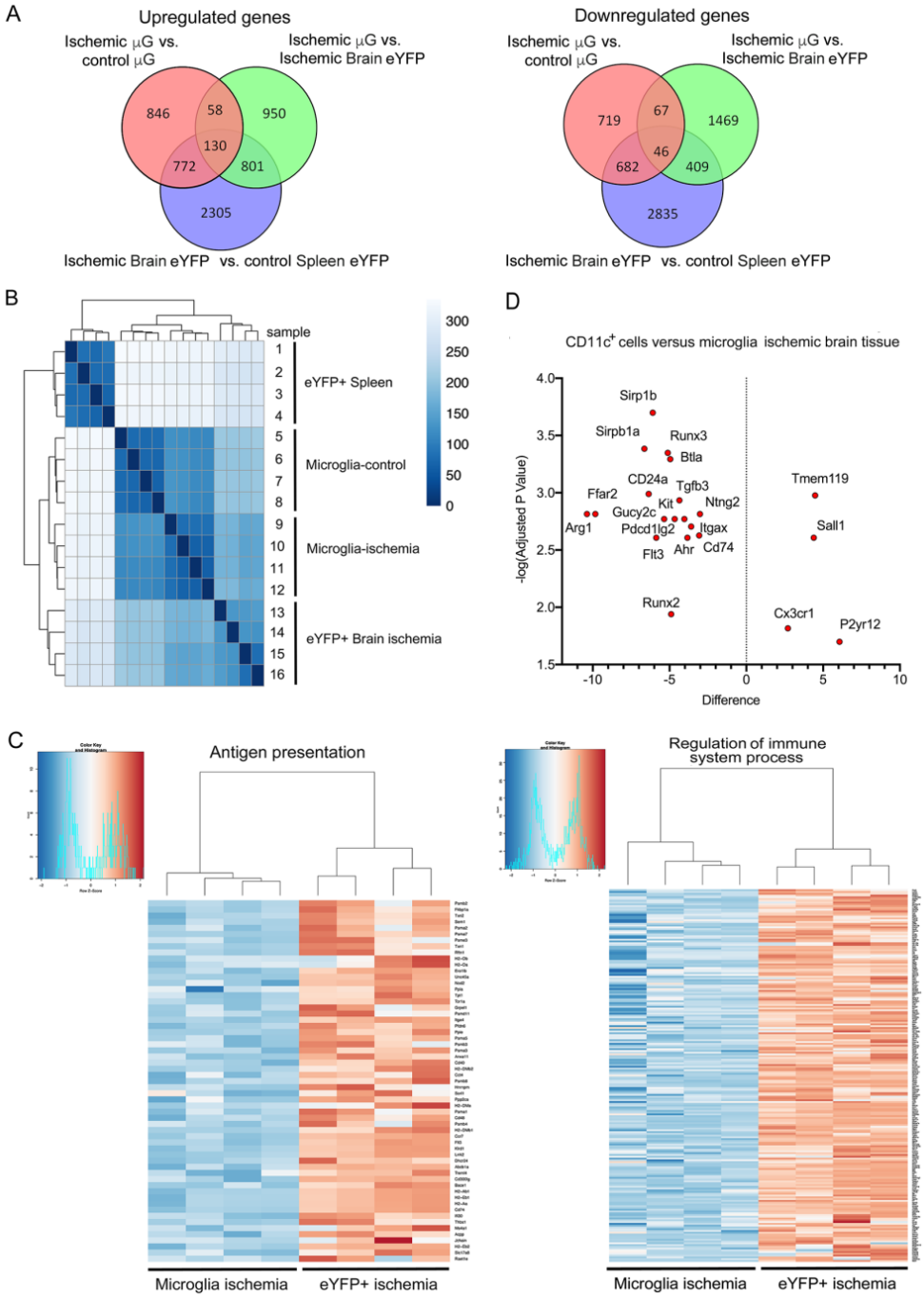
Sup. Fig. 2. **CD11c⁺ cells in the ischemic brain tissue display features different from microglia** ***Related to Fig. 1C,G***. A) *Itgax* mRNA expression (RT-PCR) in glial cell cultures obtained from postnatal brain of WT mice exposed to mouse recombinant IL-4 (50 ng/mL) or LPS (10 ng/mL). IL-4, but not LPS, induces *Itgax* expression at 8h, 24h and 48h after IL-4 (one-way ANOVA, ***p<0.001, n=9-13 samples per time point, obtained in 4 independent experiments). B) eYFP⁺ cells (green) 4 days post-ischemia (n=6) are positive for Isolectin and Iba-1 (red); To-Pro3 stained nuclei (blue) are also shown in the corresponding merged images. However, some CD11c-eYFP⁺ cells are negative for P2YR12 (blue). Scale bar: 10 µm. C) We FACS sorted eYFP⁺ cells from the brain of CD11c-eYFP mice, and microglia (µG) from the brain of CX3CR1cre^{ERT2}:Rosa26-tdT mice under control conditions and 4 days post-ischemia. We obtained mRNA from the sorted cells and carried out RT-PCR. Values correspond to cells from ischemic mice and are expressed as fold vs. gene expression in microglia from control brain. Expression of typical microglial genes, *Tmem119, Sall1*, and *P2yr12* is lower in CD11c⁺ cells compared to microglia (Mann-Whitney test, *p=0.029, n=4 mice per group). Bars show the mean±SEM and symbols show the individual values per each mouse.

Supplementary Fig. S3



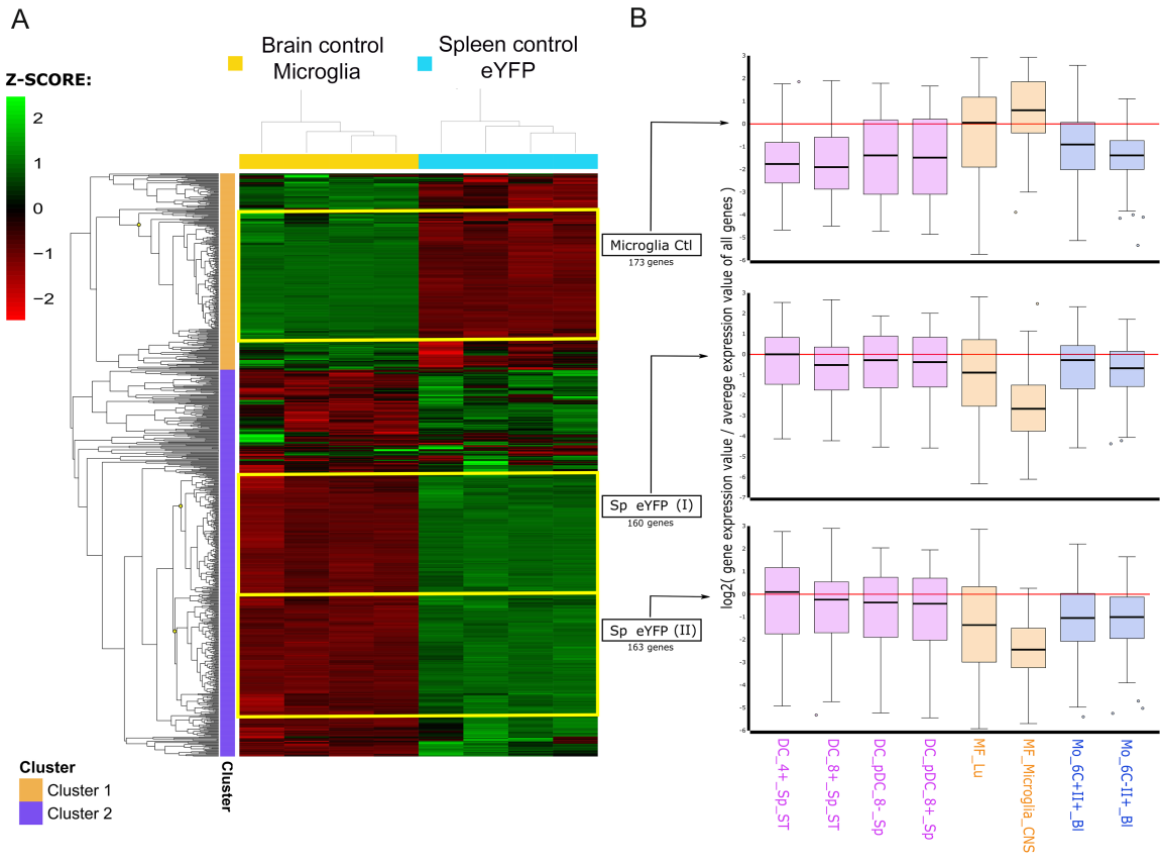
Sup. Fig. S3. Gating strategy for FACS of microglia and CD11c-eYFP⁺ cells. ***Related to Fig. 2A.*** A) Gating strategy for sorting microglia from the brain of CX3CR1cre^{ERT2}:Rosa26-tdT mice. We checked by flow cytometry the purity of sorted microglia: 98.3% (n=1) for control microglia, and 93.9±1.4% (n=2) for ischemic microglia. B) Gating strategy for sorting eYFP^{+} cells from the ischemic brain of CD11c-eYFP mice**.** Purity of eYFP^{+} cells: 86.4%, n=1, as assessed by flow cytometry.

Supplementary Fig. S4



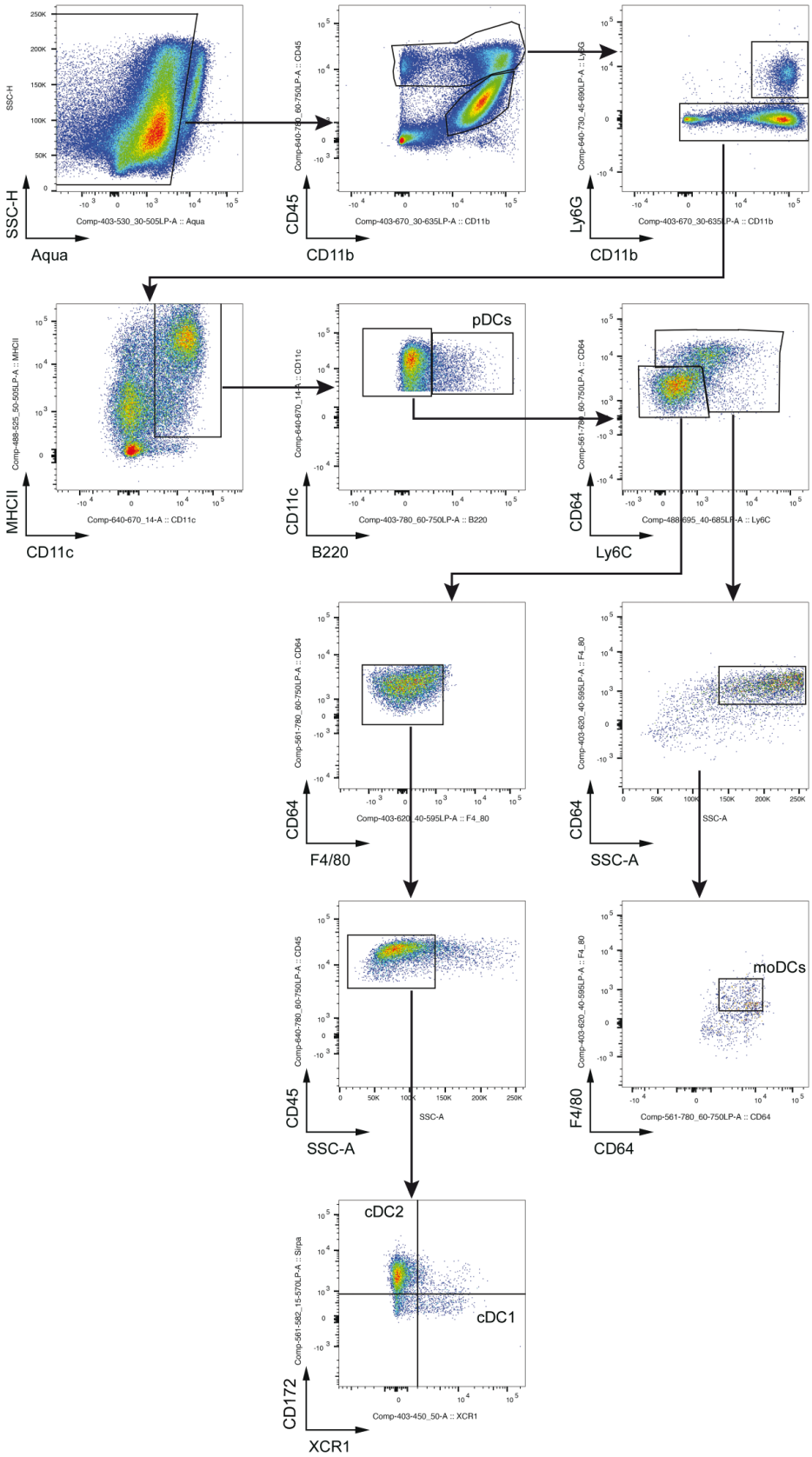
Sup. Fig. S4. Analysis of genes differentially expressed in the various cell populations. ***Related to Fig. 2B, C.*** A) We compared gene expression in microglia (μG) versus eYFP$^+$ cells of the ischemic brain (4 days post-ischemia), and the latter cells with spleen eYFP$^+$ cells. We also compared ischemic versus control microglia. The Venn diagrams show the distribution of upregulated and downregulated genes in each comparison and the overlap between them B) The dendrogram shows separation between the different samples of each cell group (n=4 per group) according to unsupervised analysis. C) Heatmaps of the GO terms: *Antigen presentation* and *Regulation of Immune system process* in the comparison between microglia and eYFP$^+$ cells, both obtained from the ischemic brain tissue. Genes in these terms are significantly overrepresented (red) in eYFP$^+$ cells versus microglia (Log2FC >1.5 adjusted p value <0.001). D) From the RNA-Seq analysis, we selected several genes differentially expressed between CD11c-eYFP$^+$ cells and microglia of the ischemic brain tissue for independent validation by RT-PCR. The selected group of genes clearly separated microglia genes from DC genes, as illustrated in the Volcano plot.

Supplementary Fig. S5



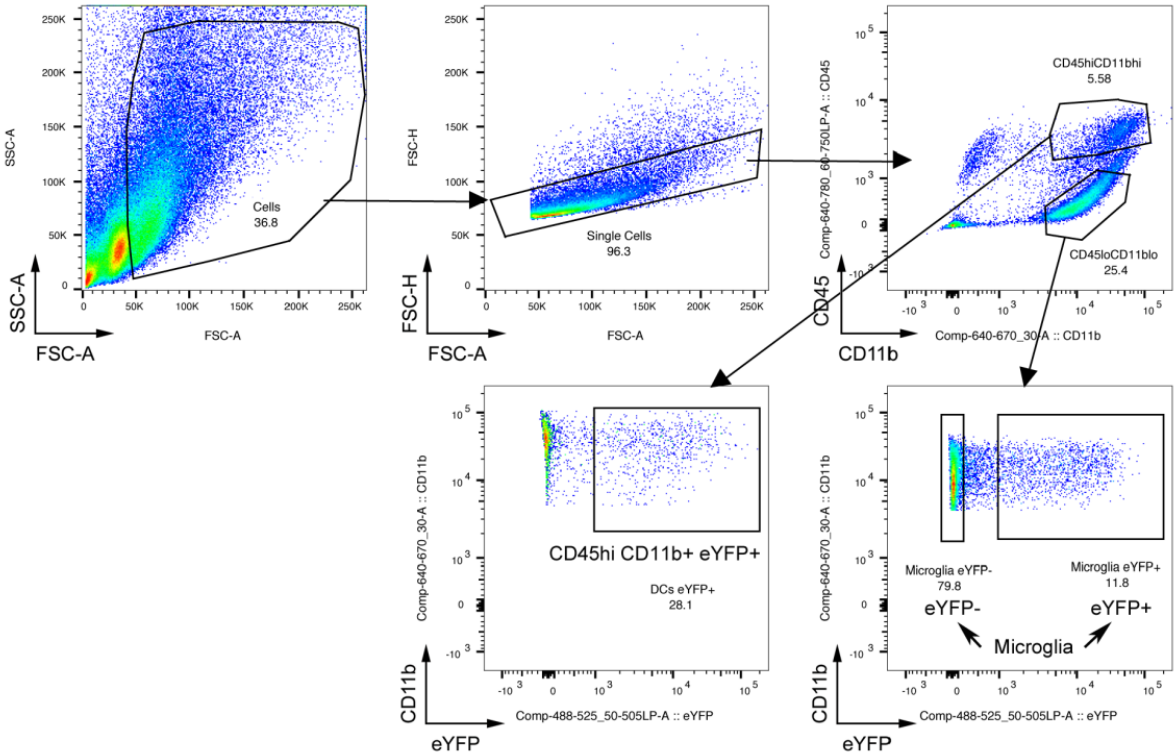
Sup. Fig. S5. Comparison of the transcriptomic data of control brain microglia and control spleen CD11c-eYFP⁺ cells with ImmGen reference cell population expression profiles. ***Related to Fig. 2C, D.*** A) We compared our RNA-Seq data of Control brain microglia (n=4) and Control spleen eYFP⁺ cells (n=4) with ImmGen reference cell populations. The comparison retrieved 787 genes that were used to construct a heatmap representation and perform hierarchical clustering. The representation includes two natural clusters found by unsupervised clustering. Clusters correspond to genes upregulated (green) or downregulated (red) in each cell group. Three subclusters were selected due to their differential expression amongst the two studied groups (each marked with a yellow square on the heatmap). Subcluster *Microglia Ctl*. is upregulated in control brain microglia and contains 173 genes. Subclusters *Sp eYFP (I)* and *(II)* are upregulated in control spleen eYFP⁺ cells and contain 160 and 163 genes, respectively. B) The expression of the genes included in each subcluster was checked on the reference cell populations using the *My Geneset* tool available in ImmGen online resources. The graphs display the generated boxplots for each of the subclusters. The *Microglia Ctl* subcluster is upregulated among the ImmGen macrophage/microglia population (*MF_Microglia_CNS*) and, to a lower extent, among peripheral macrophages (*MF_Lu*). The subclusters corresponding to spleen eYFP⁺ cells termed *Sp eYFP (I)* and *Sp eYFP (II)* display a slight upregulation in ImmGen splenic CD4⁺ myeloid DCs (*DC_4+_Sp_ST*) and the remaining DC populations (*DC_8+_Sp_ST, DC_pDC_8-_Sp* and *DC_pDC_8+_Sp*), whereas they were downregulated in ImmGen CNS macrophages/microglia (*MF_Microglia_CNS)*.

Supplementary Fig. S6



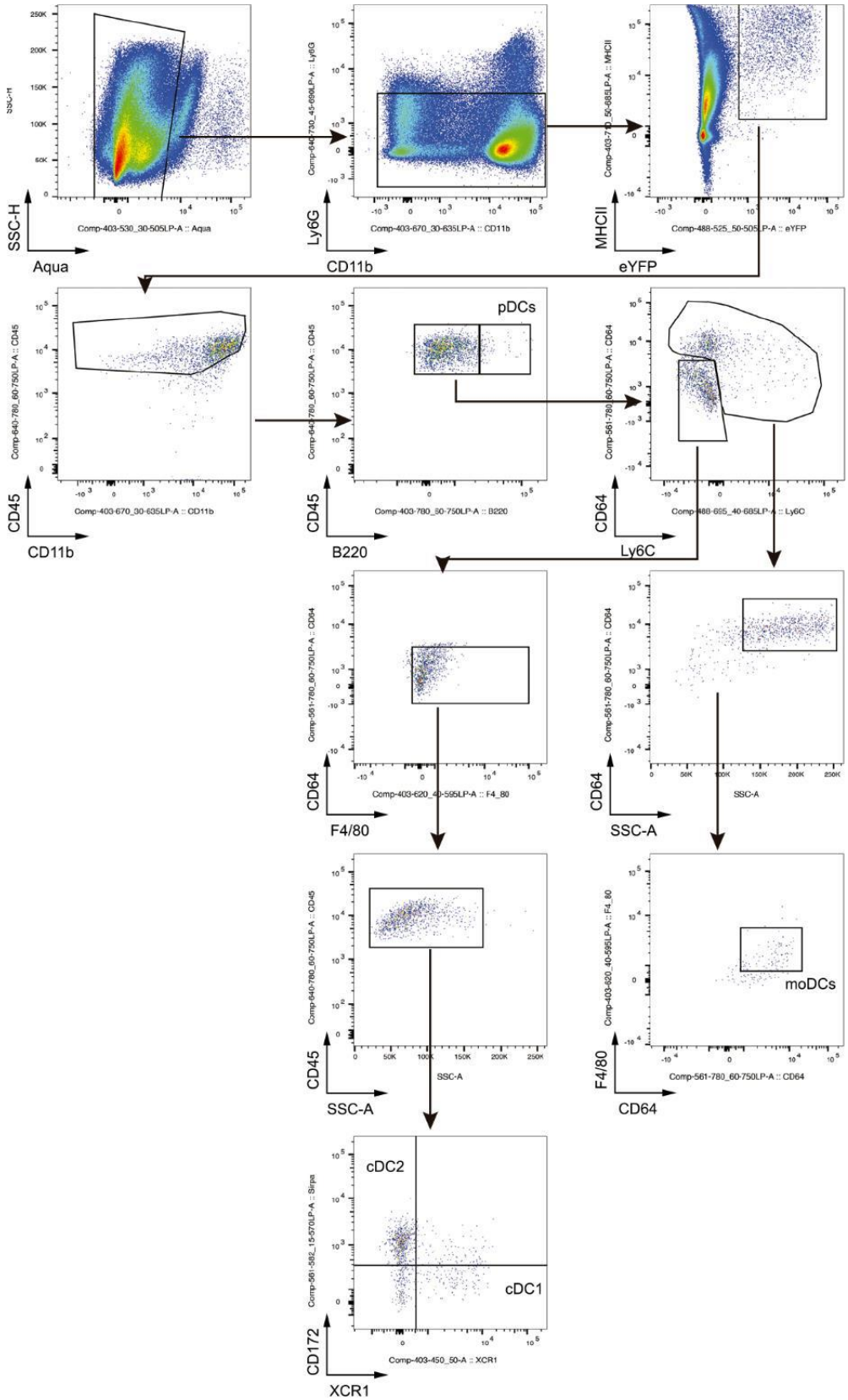
Sup. Fig. S6. Gating strategy. ***Related to Fig. 4F.*** Strategy for DC subsets in WT mouse brain four days post-ischemia.

Supplementary Fig. S7



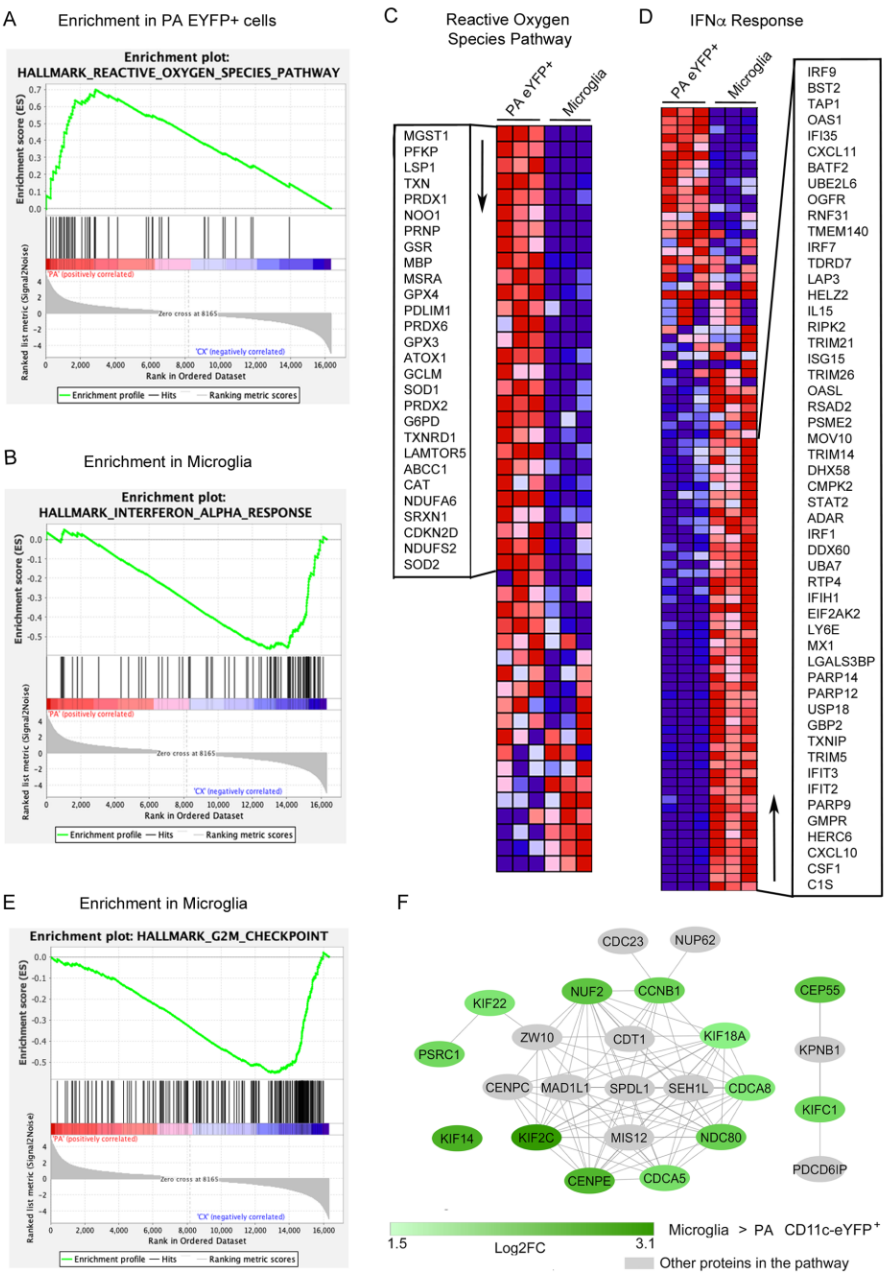
Sup. Fig. S7. Gating strategy. ***Related to Fig. 4H, I.*** Strategy for cell sorting to obtain $CD45^{lo}CD11b^{+}eYFP^{-}$ microglia, $CD45^{lo}CD11b^{+}eYFP^{+}$ microglia, and $CD45^{hi}CD11b^{+}eYFP^{+}$ cells for T cell proliferation assays.

Supplementary Fig. S8



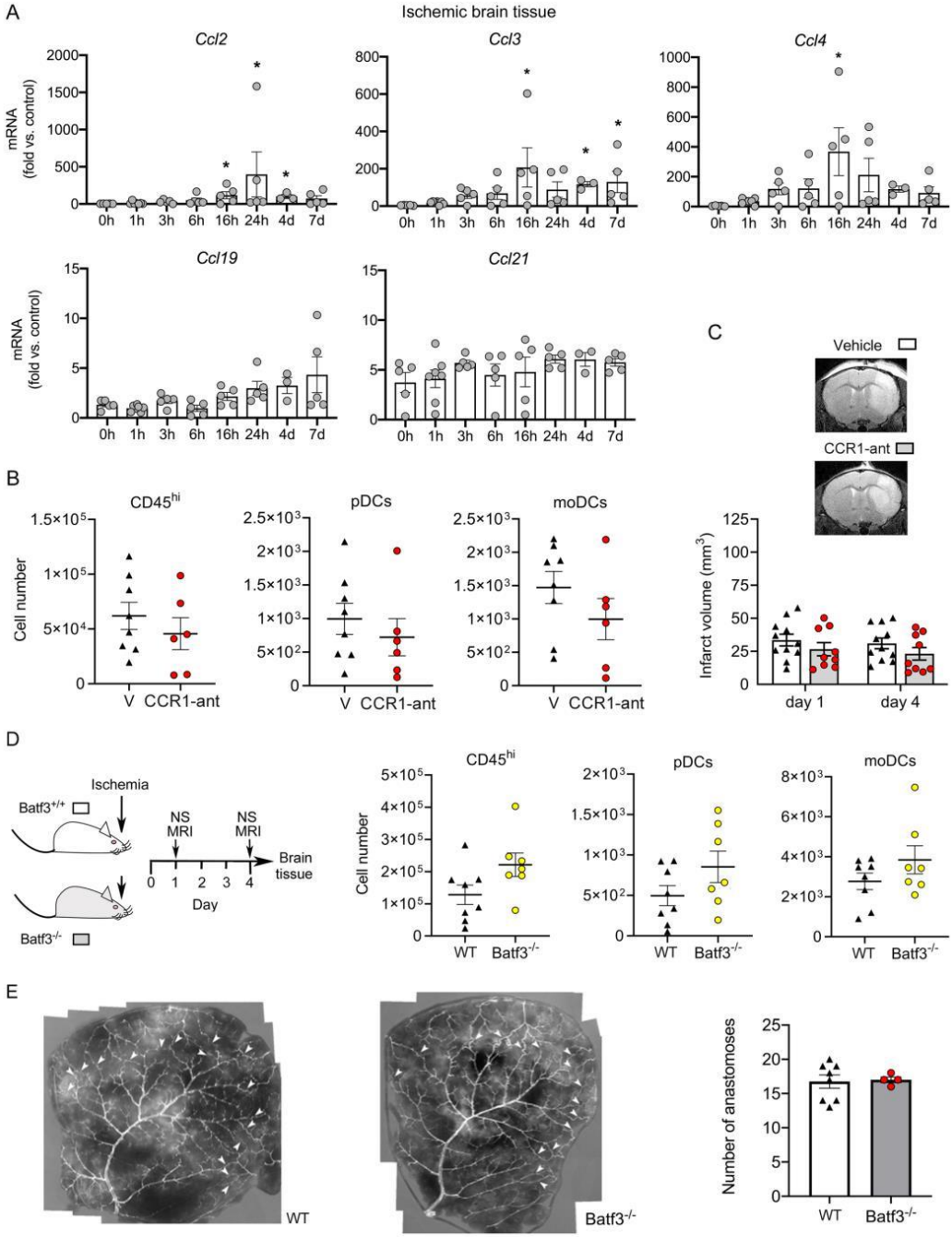
Sup. Fig. S8. Gating strategy for CD11c-eYFP⁺ DC subsets. ***Related to Fig. 5H.*** Data obtained from the brain of ischemic WT parabiotic mice, 4 days post-ischemia. The gating shows cDC1, cDC2, moDCs and pDCs.

Supplementary Fig. S9



Sup. Fig. S9. Gene Set Enrichment Analysis (GSEA) of the RNAseq of microglia vs. infiltrating eYFP⁺ cells of parabiotic wild type mice 4 days post-ischemia (PA eYFP⁺). ***Related to Fig. 5E, G.*** A) Enrichment plot showing the pathway with the highest enrichment score for PA eYFP⁺ cells, i.e. Reactive Oxygen Species (ROS). B) Respective heat map showing clustered genes in the leading edge subset of the ROS pathway. The range of colors (red, pink, light blue, dark blue) shows the range of expression values (high, moderate, low, lowest). Arrows indicate the direction of the list of genes from high towards low enrichment score. The genes listed in the heat map are those contributing to the leading-edge subset within each gene set. C) Enrichment plot showing the pathway with the highest enrichment score for Microglia, i.e. Interferon-α (IFN-α) response pathway. D) Respective heat map showing the clustered genes in the leading edge subset of the IFN-α response pathway. E) Enrichment plot in microglia showing one of several pathways with genes involved in cell proliferation, that are not enriched in infiltrating PA eYFP⁺ cells. F) Cytoscape illustration of a protein pathway representative of cell cycle-related processes (GO term: *Mitotic metaphase plate congression)* generated from genes upregulated in microglia versus infiltrating CD11c-eYFP⁺ cells, and other molecules in the pathway not differentially expressed in our samples (grey).

Supplementary Fig. S10



Sup. Fig. S10. In various experimental conditions: DC chemoattraction after ischemia, leukocyte infiltration, and collateral vessels. ***Related to Fig. 6, 7.*** A) Brain chemokine mRNA at several time points post-ischemia (h, hours; d, days), in addition to Ccl5 and Ccl8 mRNA shown in Fig. 6C, (n=3-7 mice per time point), Kruskal-Wallis & Dunn's test (*p<0.05). B) Mice received daily CCR1 antagonist J113863 (CCR1-ant)(10 mg/Kg, i.p.) or vehicle (V) starting 1 day post-ischemia and up to day 3. Brain tissue was studied at day 4 by flow cytometry in mice treated with CCR1-ant (n=6) or V (n=8). Treatment did not affect the number of CD45^{hi} cells, pDC or moDCs in the ischemic tissue. C) Representative MRI (Turbo-RARE) images of the brain lesion 1 day post-ischemia, i.e. before treatment, show similar lesion volume in both groups. Four mice per group died and were excluded. The treatment did not modify the volume of infarction at day 4 post-ischemia (V n=11 mice; CCR1-ant n=10 mice). D) Brain tissue of *Batf3*$^{-/-}$ (n=8) and WT (n=8) mice 4 days post-ischemia showed no group differences in CD45^{hi} cells, pDCs or moDCs. E) *Batf3*$^{-/-}$ mice have a cerebrovascular anatomy similar to that of WT mice. Counting the number of vascular anastomoses (arrowheads) on the brain surface showed comparable values in *Batf3*$^{-/-}$ mice (n=4) and WT mice (n=8). Measures were carried out in a blinded fashion. An independent researcher validated the results with a significant inter-observer correlation (**p<0.007, Pearson r=0.73).

SUPPLEMENTARY TABLES

Sup. Table S1. Functional annotation clustering showing functions overrepresented in CD11c-eYFP⁺ cells vs. microglia. ***Related to Fig. 2C***

	Enrichment Score	Annotation Clusters	Count	%	Fold Enrichment	Benjamini	FDR
1	8,776	Cell-cell adherens junctions	67	3,59	2,428	2,14E-09	2,60E-08
		Cadherin binding in cell-cell adhesion	60	3,21	2,411	1,80E-07	4,31E-07
		Cell-cell adhesion	39	2,09	2,347	1,60E-03	1,97E-03
2	2,834	Phosphorylation	85	4,55	1,579	1,68E-02	4,86E-02
		Kinase activity	90	4,82	1,497	1,38E-02	1,67E-01
		Transferase activity	172	9,21	1,310	1,52E-02	2,19E-01
		Protein autophosphorylation	32	1,71	1,989	7,53E-02	5,44E-01
		Protein phosphorylation	74	3,96	1,461	1,50E-01	1,71E+00
		Protein kinase activity	68	3,64	1,436	1,35E-01	3,59E+00
		Protein serine/threonine kinase activity	54	2,89	1,415	3,08E-01	1,35E+01
		ATP binding	148	7,92	1,101	8,40E-01	8,78E+01
		Nucleotide binding	187	10,01	1,083	8,63E-01	9,20E+01
3	2,615	MHC class II protein complex	10	0,54	9,543	3,74E-06	7,56E-05
		Intestinal immune network for IgA produ.	17	0,91	4,194	1,96E-04	9,39E-04
		Antigen process /presentation peptide MHC II	9	0,48	7,311	5,94E-03	1,22E-02
		Antigen proc. pres. peptide or polysac MHC II	7	0,37	8,845	1,73E-02	5,73E-02
		Rheumatoid arthritis	21	1,12	2,654	3,95E-03	9,48E-02
		MHC class II protein complex binding	8	0,43	5,980	1,56E-02	2,45E-01
		Tuberculosis	33	1,77	1,943	1,34E-02	3,87E-01
		Leishmaniasis	16	0,86	2,591	2,67E-02	1,16E+00
		Toxoplasmosis	23	1,23	2,109	2,70E-02	1,30E+00
		Inflammatory bowel disease	15	0,80	2,635	2,59E-02	1,50E+00
		Asthma	9	0,48	3,886	2,52E-02	1,70E+00
		Antigen processing and presentation	17	0,91	2,148	6,08E-02	5,84E+00
		Antigen processing and presentation	11	0,59	2,317	6,12E-01	2,89E+01
		Staphylococcus aureus infection	11	0,59	2,280	1,73E-01	2,25E+01
		Chaperone mediated protein folding cofactor	5	0,27	4,374	6,64E-01	3,46E+01
		Allograft rejection	10	0,54	1,850	3,66E-01	6,93E+01
		Influenza A	23	1,23	1,394	3,80E-01	7,42E+01
		Graft-versus-host disease	9	0,48	1,793	4,17E-01	8,23E+01
		Viral myocarditis	12	0,64	1,574	4,36E-01	8,54E+01
		Autoimmune thyroid disease	11	0,59	1,605	4,43E-01	8,67E+01
		Type I diabetes mellitus	9	0,48	1,504	5,88E-01	9,75E+01
		Phagosome	20	1,07	1,191	6,53E-01	9,93E+01
		Systemic lupus erythematosus	16	0,86	1,128	7,50E-01	1,00E+02
4	2,448	Proteasome core complex	9	0,48	5,153	6,00E-03	2,44E-01
		Threonine-type endopeptidase activity	9	0,48	4,387	4,47E-02	9,26E-01
		Proteasome	12	0,64	2,763	4,41E-02	3,82E+00
		Antigen proc. pres. exogenous peptide MHC I	9	0,48	3,412	2,87E-01	6,45E+00
		Proteasome core complex, α-subunit complex	5	0,27	6,362	7,39E-02	7,48E+00
		Proteolysis cellular protein catabolic process	12	0,64	2,394	5,10E-01	1,71E+01
		Endopeptidase activity	18	0,96	1,802	4,49E-01	2,90E+01
		Proteasome complex	12	0,64	2,082	2,25E-01	3,38E+01

Sup. Table S2. Enrichment analysis in Parabiotic CD11c-eYFP^{+} cells vs. microglia in the ischemic brain tissue of wild type mice. ***Related to Fig. 5E.***

gene_name	log2FoldChange	padj
Htr7	12,66	4,14E-21
Fam83f	11,34	2,09E-18
Cdh1	10,99	2,43E-31
Cdh17	10,76	4,49E-09
Chil3	10,69	3,13E-19
S100a9	10,58	4,29E-05
Ffar2	10,42	7,37E-15
F7	10,30	3,11E-39
B3gnt5	10,18	7,21E-18
F10	10,14	2,90E-15
Ltf	10,08	1,84E-06
H2-Eb2	9,81	2,69E-08
Ifitm1	9,70	3,61E-50
P2ry10	9,67	8,40E-62
Slc16a14	9,59	2,75E-11
Gm9733	9,58	1,33E-12
Napsa	9,50	1,12E-92
S100a8	9,45	2,28E-07
Il1r2	9,44	2,50E-58
Lad1	9,38	1,56E-06
Epcam	9,33	4,27E-17
Spsb4	9,17	5,28E-12
Sgms2	9,09	2,93E-12
Gcsam	8,88	1,48E-12
Gpr171	8,88	6,49E-63
Pkp3	8,79	1,95E-17
Zdhhc15	8,79	4,06E-11
Dpp4	8,75	5,81E-45
Wnt11	8,75	6,17E-11
Cd24a	8,75	1,26E-62
Anxa1	8,66	8,37E-123
Tnfsf4	8,59	5,44E-18
Klri1	8,58	9,96E-09
Gpr141	8,48	7,10E-44
Gpr33	8,43	7,02E-09
Cd177	8,41	1,82E-17
Kcne3	8,19	1,19E-08
Ocstamp	8,14	3,79E-35
Ramp3	8,07	7,69E-16
Ppbp	8,07	5,90E-06
Acpp	8,04	3,98E-17
Scin	8,02	5,66E-10
Dcstamp	8,02	8,99E-38
Apol7c	7,93	8,99E-07
Ceacam19	7,93	6,47E-08
Itgb7	7,92	2,15E-42
Klrk1	7,91	4,70E-44
Alpk2	7,85	1,72E-07
Plbd1	7,85	2,36E-65
Ly6c2	7,83	5,05E-32

Sup. Table S3. Processes highlighted by enrichment analysis in microglia (MG) and/or parabiotic cD11c-eYFP^{+} cells (PA-eYFP) in the ischemic brain tissue. ***Related to Fig. 5E.***

	MG	PA-eYFP
Cell cycle	✓	
- Cell cycle progression	✓	
- Cell division	✓	
- Chromosome formation	✓	
- DNA repair mechanisms	✓	
Neuronal function	✓	
- Cognitive function	✓	
- Synaptic function	✓	
Immune response		✓
- General immune response		✓
- Innate immunity		✓
- Inflammation	✓	✓
- Adaptive immunity		✓
- Cytokine secretion		✓
- Response to toxic substance		✓
- Haematopoiesis		✓
- Leukocyte differentiation		✓
- Immunity-related diseases	✓	✓
Response to cellular stress		✓
- Oxidative stress		✓
Calcium signalling	✓	✓
Migration	✓	✓
- Motility	✓	✓
- Actin biology	✓	✓
- Immune migration and chemotaxis		✓
- Cell adhesion		✓
- Tissue invasion and metastasis		✓

	MG	PA-eYFP
Glial processes	✓	✓
- Microglia activation		✓
- Astroglia activation	✓	
Barrier integrity	✓	✓
- Cell junction	✓	✓
- Extracellular matrix	✓	✓
Hypoxia response and vascular repair	✓	✓
- Response to hypoxia		✓
- Angiogenesis	✓	✓
- Vascular remodelling	✓	✓
- Coagulation		✓
Cell development and differentiation	✓	✓
- Brain development	✓	
- Glial morphogenesis	✓	✓
- Neuronal morphogenesis	✓	
- Neuronal degeneration	✓	
- General cell development	✓	✓
Other enriched processes	✓	✓
- Cardiovascular diseases	✓	✓
- Other diseases	✓	✓
- Cancer-related pathways	✓	✓
- Signalling pathways	✓	✓
- Membrane system		✓
- Protein localization		✓
- Protein polymerization	✓	
- Thermogenesis	✓	✓

MG includes 961 genes and PA-eYFP includes 793 genes with non-corrected p value<0.001 |LOG2FC| >2
Enriched pathways: exclusive MG = 264, exclusive PA-eYFP=595, common=198

Sup. Table S4. Primers used for RT-PCR. ***Related to Star Methods***

Gene Symbol	Gene Name	Assay ID	Ref sequence	Amplicon length	Probe spans exons	Exon Boundary
Ahr	aryl-hydrocarbon receptor	Mm00478932_m1	NM_013464.4	51	yes	3-5
Arg1	arginase 1	Mm00475988_m1	NM_007482.3	65	yes	1-2
Btla	B and T lymphocyte associated	Mm00616981_m1	NM_001037719.2	71	yes	5-6
Ccl2	chemokine (C-C motif) ligand 2	Mm00441242_m1	NM_011333.3	74	yes	1-2
Ccl3	chemokine (C-C motif) ligand 3	Mm00441258_m1	NM_011337.2	78	yes	1-2
Ccl4	chemokine (C-C motif) ligand 4	Mm00443111_m1	NM_013652.2	70	yes	1-2
Ccl5	chemokine (C-C motif) ligand 5	Mm01302427_m1	NM_013653.3	103	yes	1-2
Ccl7	chemokine (C-C motif) ligand 7	Mm00443113_m1	NM_013654.3	122	yes	1-2
Ccl8	chemokine (C-C motif) ligand 8	Mm01297183_m1	NM_021443.3	61	yes	1-2
Ccl19	chemokine (C-C motif) ligand 19	Mm00839966_g1	NM_011888.2	70	yes	1-2
Ccl21	chemokine (C-C motif) ligand 21	Mm03646971_gH	NM_011124.4	91	yes	3-4
Ccr1	chemokine (C-C motif) receptor 1	Mm01216147_m1	NM_009912.4	149	yes	1-2
Ccr2	chemokine (C-C motif) receptor 2	Mm00438270_m1	NM_009915.2	100	yes	2-3
Ccr7	chemokine (C-C motif) receptor 7	Mm01301785_m1	NM_007719.2	78	yes	2-3
Cd24a	CD24a antigen	Mm01191887_g1	NM_009846.2	58	yes	1-2
Cd74	CD74 antigen	Mm00658576_m1	NM_001042605.1	118	yes	1-2
Cx3cr1	chemokine (C-X3-C motif) receptor 1	Mm00438354_m1	NM_009987.4	92	yes	1-2
Ffar2	free fatty acid receptor 2	Mm01176528_g1	NM_001168509.1	81	yes	2-3
Flt3	FMS-like tyrosine kinase 3	Mm00439016_m1	NM_010229.2	108	yes	22-23
Flt3lg	FMS-like tyrosine kinase 3 ligand	Mm00442801_m1	NM_013520.3	95	yes	6-7
Gucy2c	guanylate cyclase 2c	Mm01267705_m1	NM_001127318.1	65	yes	24-25
Hrpt1	hypoxanthine guanine phosphoribosyl transferase	Mm03024075_m1	NM_013556.2	131	yes	2-3
Itgax	integrin alpha X	Mm00498701_m1	NM_021334.2	94	yes	17-18
Kit	kit oncogene	Mm00445212_m1	NM_001122733.1	71	yes	7-8
Ntng2	netrin G2	Mm01325566_m1	AB052336.1 (GenBank)	65	yes	5-6
Pdcd1lg2	programmed cell death 1 ligand 2	Mm00451734_m1	NM_021396.2	95	yes	4-5
P2ry12	purinergic receptor P2Y, G-protein coupled 12	Mm01289605_m1	NM_027571.3	74	yes	2-3
Runx2	runt related transcription factor 2	Mm00501584_m1	NM_001145920.2	91	yes	6-7
Runx3	runt related transcription factor 3	Mm00490666_m1	NM_019732.2	73	yes	4-5
Sall1	sal-like 1	Mm07297700_m1	NM_021390.3	102	yes	3-4
Sirpb1a	signal-regulatory protein beta 1A	Mm02344810_m1	NM_001002898.1	104	yes	4-5
Sirpb1b	signal-regulatory protein beta 1B	Mm02344809_g1	NM_001173460.1	133	yes	3-4
Tgfb3	transforming growth factor, beta 3	Mm00436960_m1	NM_009368.3	60	yes	3-4
Tmem119	transmembrane protein 119	Mm00525305_m1	NM_146162.2	72	yes	1-2

Discussion

The central nervous system has been historically considered an immunologically privileged space, mostly due to several pieces of evidence that pointed at his capacity to withstand tumour, tissue or microorganism injection in the parenchyma, without overt rejection by the immune system (Galea et al., 2007).
In the first half of the 1900s this idea was further corroborated by studies that revealed the structure and function of the BBB, attributing to this structure the most important role – hence the name: barrier, not gate (Galea et al., 2007).
It is clear now that the peculiar immunological features of the CNS are not only due to the physical separation of the parenchyma from the rest of the organism, but also to the strict control of the immune response in the parenchyma and adjacent spaces, namely the choroid plexus, the meninges and the cerebrospinal fluid that bathes them.
Early evidence pointed at the brain afferent arm of adaptive immunity, antigen presentation, as the main responsible of the maintenance of the immune privilege, while the efferent arm, the response by lymphocytes, behaved more conventionally (Matyszak and Perry, 1995; Medawar, 1948).
Upon inflammatory stimuli, the immune privilege of the brain is perturbed through several mechanisms, all of which manifest after stroke: BBB disruption, local cytokine and chemokine production, infiltration of peripheral leukocytes and increased brain-derived antigen presentation (Chamorro et al., 2012).
Knowing how antigen-presenting cells are one of the most important players in the maintenance of this delicate equilibrium in the CNS, it is only logical to assume they can play an equally significant role in the pathophysiology of stroke.

Possibly due to the late appearance of specific experimental tools, perhaps also due to the persistence in part of the scientific community of the old idea of immune privilege as an absolute concept, or to the relatively late description of DCs as separate professional APCs (Steinman and Cohn, 1973), the investigation on dendritic cell involvement in brain diseases has gained traction later compared with other immune cell types.
Furthermore, research on DCs has been historically – for evidently good reasons – focused on their role as APCs, and on the mechanisms that directly depend on the interaction of their antigen presentation machinery with the T cell receptor machinery on lymphocytes, which represents the base of the adaptive immune response.
However, several pieces of evidence have been recently emerging, showing how DCs can modulate the immune environment through innate mechanisms, such as their capacity to produce cytokines in response to PRR stimulation. The innate role of DCs has been associated in different settings with the involvement of γδ T cells, since

these cells do not necessarily need antigen presentation to become activated, making this axis crucial in the rapid response to pathology (Gelderblom et al., 2012b), or even in the maintenance of homeostasis (Martínez-López et al., 2019).
The relatively recent emergence of clear distinctions between different subsets of DCs, and the recent proposals for unified nomenclatures (Guilliams et al., 2014; Guilliams and van de Laar, 2015) have also made evident the necessity to investigate the involvement of DCs in diseases not as a single population, but keeping in mind the unique characteristics of their subsets.
Especially in brain diseases, this has rarely been the case, with most of the data coming from general studies of DCs, mostly identified by their expression of few surface markers, usually CD11c and MHCII. This does not reduce the validity of the studies, but partially limits the interpretation of the results.

In this context, and for some experiments before part of the most recent information was available, we set out to contribute to the knowledge about the involvement of DCs in stroke by improving the molecular definition of brain DCs and by investigating the role of the two main subtypes of conventional DCs.

I will hereby separately resume and discuss the results derived from each of the scientific papers presented in this dissertation, and then proceed to a global discussion.

DISCUSSION OF ARTICLE #1

Gelderblom, M.*, **Gallizioli, M.***, et al. IL-23 (Interleukin-23)–producing conventional dendritic cells control the detrimental IL-17 (Interleukin-17) response in stroke. *Stroke* (2018) * *indicates equal contribution*

In this paper, we demonstrated a crucial role of cDC2 cells in the early phase of post-stroke inflammation by regulation of the activity of γδ T cells, which in turn promotes the infiltration of neutrophils.

The conceptual base of the first paper that I present in this dissertation was set in a previous work by Gelderlom et al., where they demonstrated the importance of the involvement of T cells in stroke not only as effector cells, but also as mediators of further inflammatory signals able to attract and/or activate other cell types to the ischaemic tissue (Gelderblom et al., 2012b). This study showed an interesting synergistic mechanism by which the IFN-γ produced by $CD4^+$ T cells stimulated the production of TNF-α by brain infiltrating macrophages and this, together with IL-17 secreted by γδ T cells, led to the infiltration of neutrophils to the injured brain tissue. Analysis of the infiltration of cells in the ischaemic brains of lymphocyte-deficient *Rag1*$^{-/-}$ mice and of *Tcrd*$^{-/-}$ mice, which lacked γδ T cells, showed reduced numbers of neutrophils in both strains. This reduction could only be reversed when lymphocyte-deficient mice were reconstituted with all the T cell compartment, including γδ T cells.
Since the involvement of IL-17 produced by γδ T cells in stroke brain damage had already been postulated in the delayed phase of stroke (Shichita et al., 2009), it was plausible to hypothesise that IL-17 could have been involved in the acute phase too. The study demonstrated that loss-of-function using *Il17ra*$^{-/-}$ mice, which lacked the receptor for IL-17, and early treatment with anti-IL-17 antibodies induced the same reduction of infiltrating neutrophils as the one observed in T cell-deficient mice. In this paper, Gelderblom et al. also demonstrated that neutrophils do not directly react to IL-17 signalling, since they lack IL-17 receptor, but they respond to the CXCL-1 released by astrocytes upon stimulation with IL-17 and TNF-α (Gelderblom et al., 2012b). Moreover, the neutralisation of IL-17 resulted in a protective effect, mediated both by reduced neutrophil infiltration and by MMP3 production. This work left opened the question of which mechanisms and cell types regulated the production of IL-17 by γδ T cells in the early phase of the stroke inflammatory response. And this is indeed the question that the first article presented in this dissertation meant to investigate.

Previous work described the temporal dynamics of the infiltration of peripheral cells into the ischaemic hemisphere, placing the entrance of dendritic cells at around 24h after reperfusion (Gelderblom et al., 2009). We corroborated this observation by immunofluorescence and immunohistochemistry of CD11c$^+$ cells in the ischaemic parenchyma of both mice and human samples, obtaining results compatible with previous reports (Felger et al., 2010; Reichmann et al., 2002).
Given that also T lymphocytes start to infiltrate the ischaemic brain around 24h after reperfusion (Gelderblom et al., 2009), we pursued the exploration of the relationship between the early infiltrating DCs and the cellular axis comprising γδ T cells, astrocytes and neutrophils.

The first piece of evidence of the possible relationship between DCs and neutrophils was obtained in the CD11c.DOG mouse model of dendritic cell depletion (Jung et al., 2002). Besides the predictable decrease in CD11c$^+$ cell infiltration, we observed a significant reduction of brain neutrophil infiltration, while the number of other infiltrating leukocytes was not significantly affected. The parallel increase in peripheral neutrophils in the blood was a further indication that reduced neutrophil infiltration could be dependent on an altered migration of these cells to the ischaemic brain, more than a direct alteration of their development. Moreover, we observed reduced infarct sizes and mortality, together with milder neurological symptoms at day 1 after ischaemia in CD11c.DOG mice versus WT mice. This result is compatible with the extensively demonstrated deleterious effect of early neutrophil infiltration in the ischaemic brain, where they release proteolytic enzymes and reactive oxygen species that directly contribute to the injury, and pro-inflammatory cytokines and chemokines, which amplify the inflammatory response (Neumann et al., 2015; Otxoa-de-Amezaga et al., 2019a; Perez-de-Puig et al., 2015).

We then moved to analyse another component of the axis by observing that the depletion of CD11c$^+$ cells led to a significant decrease of the expression of IL-17 in infiltrated γδ T cells already at 24h after stroke, while no alterations were visible in the αβ compartment. We also observed decreased levels of *Cxcl1* mRNA in whole brain homogenates of CD11c-depleted ischaemic mice. These results were coherent with the previous work by Gelderblom et al. and confirmed that indeed DCs may be situated upstream of the γδ T cell-astrocyte-neutrophil axis (Gelderblom et al., 2012b). DCs rapidly interacted with γδ T cells, which for their peculiar characteristics are poised to promptly react to inflammatory stimuli, even without passing through antigen presentation-dependent stimulation (Chien et al., 2014).
A previous study could not ascribe the early detrimental effects of T cells observed in early phases of stroke either to adaptive immune reactions or to thrombogenic

mechanisms (Kleinschnitz et al., 2010). Since the study did not explore brain infiltration of myeloid cells, it is plausible that one of the mechanisms contributing to the observed T cell-mediated deleterious effects was the attraction of neutrophils to the brain. However, contrary to our results, this study did not observe γδ T cells-dependency for ischaemia-induced brain damage, but does not exclude the involvement of IL-17 produced by other conventional T cells such as Th17 cells.

IL-17 production can have other consequences on the inflammatory response apart from the attraction of neutrophils, which was one of the first effects to be demonstrated (Kolls and Lindén, 2004). Its downstream signalling has been shown to induce production of pro-inflammatory cytokines, chemokines, metalloproteinases and anti-microbial peptides in various other inflammatory diseases (Milovanovic et al., 2020).

In the brain, the overexpression of IL-17 was able to activate glial cells and induce neuroinflammation even if the effect was milder compared with IL-6 overexpression (Campbell et al., 1993).

One of the other possible mechanisms of neutrophil-independent damage by IL-17 may be the disruption of BBB tight junctions by interaction with endothelial cells, which could contribute to facilitate inflammatory cell infiltration in the brain. This was observed in multiple sclerosis patients, but could also be happening in the setting of brain ischaemia (Kebir et al., 2007).

In multiple sclerosis patients, the serum concentration of IL-17 is significantly higher than in healthy subjects and it correlates with disease development. In fact, neutralization of IL-17 or its genetic absence have been shown to attenuate EAE severity (Milovanovic et al., 2020). However, in the EAE model and in multiple sclerosis, the activity of IL-17 seems to be more dependent on Th17 induction compared with other inflammatory processes. In fact, in this model, a role for $CD11c^+$ as gatekeeper cells has been proposed, since they have been shown to interact with Th17 cells, favouring IL-17 production and parenchyma invasion (Paterka et al., 2016). While in our ischaemia model we focused on the γδ T cell-derived IL-17 and its effect on astrocytes and neutrophils, we cannot exclude that Th17 cells may also be playing a role, since in our experiments we transferred whole bone marrow or unfractionated T cells.

Sutton et al. actually propose a cooperative mechanism between γδ T cells and Th17 cells, which could mediate the EAE autoimmune disease. They observed that *Tcrd*$^{-/-}$ mice, lacking γδ T cells, not only showed an overall reduction of *Il17* mRNA, but also specifically a reduction of *Il17* produced by antigen-specific $CD4^+$ Th17 cells (Sutton et al., 2009).

Recently, an increased proportion of peripheral Th17 cells was observed in ischaemic stroke patients compared with controls, together with a reduction of Treg

cells (Dolati et al., 2018). In stroke patients, IL-17 expression increased both in blood mononuclear cells and in post-mortem brain samples (Waisman et al., 2015). However, Shichita et al. concluded that in more delayed phases of experimental stroke the main source of IL-17 were γδ T cells, not $CD4^+$ T cells (Shichita et al., 2009).

Another important point that could be raised about our study is the identity of the γδ T cells that we observed infiltrating the brain. They express the IL-23 receptor and the Vγ6 segment of the γδ TCR, and their infiltration has been demonstrated to depend on CCR6 functionality (Arunachalam et al., 2017). Classically, γδ T cells that produce IL-17 within 24h after infection are considered natural γδ T cells, and they have been observed to be able to produce IL-17 even without the explicit induction of an immune response (Chien et al., 2013). These cells usually reside in peripheral epithelial and mucosal tissues, but they have been recently observed in the meninges of mice at steady state, where they account for basically all the IL-17-producing cells, and from where they promote short-term memory through modulation of brain-derived neurotrophic factor (BDNF) production (Ribeiro et al., 2019). This recent observation makes it plausible to hypothesise that the γδ T cells we observed infiltrating the brain upon ischaemia may be natural γδ T cell rapidly infiltrating from the meninges and swiftly upregulating their IL-17 expression.

All in all, it is quite clear that the evidence supporting specific γδ T cell-mediated mechanisms in stroke is neither extensive nor particularly strong, making our results a relevant step further in the understanding of these phenomena.

Perhaps even more significant are our results that attribute the stimulation of γδ T cells to IL-23 produced by DCs during the early response to the ischaemic insult.

The influence of IL-23 on the production of IL-17 by different subsets of T cells is widely accepted (Croxford et al., 2012), and the observation that almost 80% of infiltrating IL-17A$^+$ γδ T cells in our model expressed the IL-23 receptor led us to investigate whether IL-23 could be the prime mover of the downstream γδ T cell-astrocyte-neutrophil chain reaction observed during the early phases of stroke (Gelderblom et al., 2012b).

We observed that the absence of IL-23 receptor in *Il23r*$^{-/-}$ mice led to significant reduction of the frequency of IL-17A$^+$ γδ T cells, *Cxcl1* expression, neutrophil infiltration and infarct size compared with WT littermates. We were able to ascribe the effect of IL-23 receptor deficiency specifically to T cells, by transferring unfractionated T cells from *Il23r*$^{-/-}$ mice to *Rag1*$^{-/-}$ mice. We found that neutrophil infiltration was reduced in *Rag1*$^{-/-}$ mice that received *Il23r*$^{-/-}$ T cells compared with *Il23r*$^{+/+}$-reconstituted *Rag1*$^{-/-}$ mice.

The recent developments in DC subpopulation identification and nomenclature organization (Guilliams et al., 2016; Guilliams et al., 2014; Guilliams and van de Laar, 2015) led us to try to describe the early infiltration dynamics of various DC subsets after stroke, and to try to identify the main source of IL-23 in the early phase of leukocyte infiltration.

To this aim, we established a fairly comprehensive flow cytometry panel for the analysis of DCs in mouse brain tissue. With this tool, we were able to demonstrate that conventional DCs were the most prominent subpopulation at all time points analysed and that 24h was the earliest time point when significant infiltration started to be evident. We could also demonstrate that cDC2 are significantly more abundant than cDC1 in the brain of sham operated mice, and maintained higher numbers throughout all time points analysed, showing a significant peak of infiltration at day 3 after stroke.

Due to the relative abundance of cDC2 in the brain, and due to the fact that these cells were shown to control the mucosal IL-17 cytokine response (Schlitzer et al., 2013), we focused on them as possible source of IL-23. We isolated them by FACS from ischaemic brains at early time points, together with macrophages and microglia. cDC2 turned out to be the main producers of *Il23p19* transcript both at 12h and 24h after reperfusion. The expression of IL-23 was brain-specific, since spleen cDC2 displayed significantly lower expression, possibly indicating an activation-induced or environment-driven production.

To further underscore the importance of peripheral infiltrating cells in the production of IL-23 in the ischaemic brain, we also generated bone marrow chimeric mice. We observed that WT animals reconstituted with *Il23p19*$^{-/-}$ bone marrow showed a significantly reduced neutrophil infiltration compared with *Il23p19*$^{-/-}$ reconstituted with WT bone marrow. This result is compatible with previous conclusions by Shichita et al. which described infiltrating macrophages as main source of IL-23, possibly because of a less precise flow cytometry gating strategy, which included DCs in the CD45^{hi}CD11b^{+} gate they used to define macrophages (Shichita et al., 2009). In addition, chimeric WT mice receiving bone marrow from *Il23p19*$^{-/-}$ mice showed reduced number of neutrophils compared with chimeric *Il23p19*$^{-/-}$ mice that received bone marrow from WT mice. Moreover, we observed a further reduction of neutrophil infiltration in *Il23p19*$^{-/-}$-reconstituted *Il23p19*$^{-/-}$ mice. This result indicated that also resident microglia may be participating in neutrophil infiltration, although with a less important contribution, through IL-23-mediated mechanisms, as suggested in the setting of EAE (Becher et al., 2003) and confirmed in multiple sclerosis human samples (Li et al., 2007).

Altogether our data demonstrate the importance of peripheral infiltrating cDC2 in the activation of the γδ T cell-astrocyte-neutrophil axis, which ultimately leads to worse stroke outcome in our experimental model of ischaemia.
This highlights a potential detrimental mechanism of action of cDC2 in stroke, at least in the early response phase. This mechanism extends into the more delayed phases and keeps displaying a deleterious role (Shichita et al., 2009).
Future more precise loss-of-function approaches directed at the cDC2-specific production of IL-23 could more clearly reveal their relative importance in regulating immune reactions in stroke.
In our study, we did not investigate the upstream regulation of IL-23 production by cDC2, but it is possible that various DAMPs may stimulate these cells through PRR engagement. Among them, peroxiredoxin has been shown to induce the expression of IL-23 in bone marrow-derived DCs in TLR2- and TLR4-mediated fashion, while HMGB1 had limited activation capacity (Shichita et al., 2012).
Both these *in vitro* results and our own seem to indicate a fast antigen presentation-independent mechanism at play between cDC2 and γδ T cells, which then propagates rapidly to astrocytes and neutrophils via cytokine and chemokine signalling.
However, for γδ T cells, a fast reaction time does not necessarily mean antigen presentation independence; it is possible that these cells simply do not need clonal expansion because of higher frequency of antigen-specific γδ cells compared with αβ and that, because of this, the magnitude of their reaction reaches more rapidly a detectable threshold (Chien et al., 2013).
Of course, the IL-23-mediated mechanism may not be the only one involved in the relationship between DCs and γδ T cells. Benakis et al. demonstrated that $CD11b^{+}CD103^{+}$ cells in the small intestine can influence the IL-17 response of γδ T cells in experimental stroke (Benakis et al., 2016). They demonstrated that dysbiosis in the intestinal flora was increasing the capacity of mesenteric lymph node $CD11b^{+}CD103^{+}$ cells of inducing Treg differentiation of $CD4^{+}$ cells, which in turn, via IL-10 production, suppressed IL-17 γδ T cells production. These cells were then shown to migrate to the ischaemic brain and mediate IL-17-dependent detrimental effects. The classification of DCs carried out in their study does not allow to define the precise DC subpopulation responsible for this mechanism according to the newest nomenclature, but it is surely a conventional type, most likely cDC2.

While we focused our attention on the cDC2 subpopulation, we also observed a significantly smaller, more delayed infiltration of cDC1 cells, which could play a role in modulating the immune response in the ischaemic brain, possibly at later time points, and/or through different mechanisms.

DISCUSSION OF ARTICLE #2

Gallizioli, M., et al. Dendritic cells and microglia have non-redundant functions in the inflamed brain with protective effects of type 1 cDCs. *Cell Reports* (2020)

In this paper we differentiate resident microglia from infiltrating DCs in the setting of cerebral ischaemia, and we observe interactions between these two cell types. On the one hand, DCs display a differential transcriptional profile and are better capacitated for antigen presentation than microglia. Microglia, on the other hand, are shown to be important for the attraction of DCs to the ischaemic brain via production of chemokines, attracting in particular the cDC1 subpopulation, which displays beneficial functions.

Most DCs express CD11c, but they are not the only cells displaying CD11c expression. In particular, the identity of CD11c$^+$ cells in the brain is a quite controversial topic, since also CD11c$^+$ microglia have been reported (Dando et al., 2016; Kamphuis et al., 2016; Wlodarczyk et al., 2014). The lack of reliable and specific surface markers has historically made the differentiation of CD11c$^+$ microglia from infiltrating CD11c$^+$ DCs difficult. Taking advantage of the CD11c-eYFP mouse model, we observed the presence of eYFP$^+$ cells in the steady-state brain and a significant increase of these cells in the brain after ischaemia, both by flow cytometry and by immunofluorescence, accordingly to previous results (Bulloch et al., 2008; Felger et al., 2010). The conventional flow cytometry definition of infiltrating cells as CD45^{hi}CD11b^{hi} cells allowed us to observe that, upon treatment with FLT3L, the percentage of eYFP$^+$ cells among the infiltrating cells increased, meaning that FLT3L-sensitive DCs were infiltrating the brain, as expected from other studies (Anandasabapathy et al., 2011; Felger et al., 2010), including the other paper presented in this dissertation.
Interestingly, we observed that the ischaemic brain presents a favourable milieu for DCs, since we detected the expression of *Flt3lg* mRNA in the tissue, and more specifically we could attribute its production to astrocytes. This result is compatible with observations in models of astrocytic tumours, where the expression of both Flt3L and Flt3 were detected (Kirches et al., 2013) and where Flt3L has been successfully applied as anti-tumour immune-mediated therapy (King et al., 2011).

Some, but not all, eYFP$^+$ cells observed in the ischaemic brain shared morphological and phenotypical features with microglia, namely the stellate appearance and the Iba-1, Isolectin and P2YR12 staining. However, when comparing mRNA obtained

from isolated CX3CR1$^+$ microglia versus eYFP$^+$ cells, the latter showed lower mRNA levels of typical microglia genes such as *Sall1*, *Tmem119* and *P2yr12*.
In the steady-state brain, the vast majority of eYFP$^+$ cells are microglia, while 4 days after stroke the population of eYFP$^+$ cells includes microglia and infiltrating DCs in equal parts, according to our flow cytometry analysis.

To functionally differentiate these populations, we focused on the analysis of their antigen presentation capacity. Compared with microglia, eYFP$^+$ cells showed an overall increased expression of genes related to the MHCII antigen presentation machinery, including immunomodulatory molecules and co-stimulatory factors.
Comparing by flow cytometry the expression of MHCII molecules on the surface of infiltrating versus resident CD11c$^+$ cells, we observed that the expression by infiltrating cells was vastly superior, hinting at the scarce preparedness of both CD11c$^-$ and CD11c$^+$ microglia for MHCII-mediated antigen presentation.
As for the functional assessment of the effective stimulation of antigen-specific T cells, the results clearly indicated that infiltrating CD11c$^+$ cells were indeed superior in both MHCII-mediated and MHCI-mediated antigen presentation.
DCs are able to migrate from the brain to secondary lymphoid tissues (De Laere et al., 2018). In our model, infiltrating DCs expressed CCR7, whereas microglia, which are cells not expected to leave the brain, did not. Therefore, it is plausible that DCs, not microglia, were responsible for the priming of brain-specific naïve T cells in the cervical lymph nodes *in vivo*, as demonstrated in other animal models (Greter et al., 2005; Malo et al., 2018; Mundt et al., 2019). In our *ex vivo* experiments, we observed significantly lower, but not negligible, T cell stimulation by microglial cells compared with DCs. Given that the priming of naïve T cells by microglia has been observed in the inflamed brain of EAE mice (McMahon et al., 2005) and that our cells were isolated from the ischaemic inflammatory environment, it is possible that some low degree of rechallenging of primed T cells by microglia may be happening. Nonetheless, the characteristics of the *ex vivo* assay we used may poise T cells towards a heightened responsivity and we cannot exclude some degree of unspecific stimulation.

In the EAE model, Wlodarczyk et al. demonstrated that both CD11c$^+$ microglia and CD11c$^+$ infiltrating cells were expressing similar levels of MHCII molecules and were able to induce proliferation of primed CD4$^+$ cells. However, only the infiltrating cells, not microglia, were ultimately capable of inducing pathogenic T cell responses (Wlodarczyk et al., 2014). It is therefore possible that also microglia from the ischaemic brain could be able to prime T cells, as also suggested by a recent publication (Jin et al., 2018). Microglia are a heterogeneous population of cells

(Masuda et al., 2020), and microglia with different phenotypes and functions may exist under disease conditions. Studying the diversity of microglia after stroke may also help to find out whether certain subsets may be capable of activating T cells.

Furthermore, additional experiments with separation of naïve and memory T cells, or with previous *in vivo* antigen exposure and priming, would be useful to dissect these mechanisms, even more considering that the identity of the T cells responding to brain antigens in the early phases is not clear. In fact, several mechanisms may be at play in stimulating a T cell response, from epitope spreading (McMahon et al., 2005) to innate-like TCR-independent stimulation of circulating memory T cells (von Geldern et al., 2006). The topic of brain-derived antigen presentation by brain APCs is currently under very active investigation and has a lot of therapeutic potential for numerous neurological diseases, from the most immunologically driven to the ones where the involvement of immune cells is not so clear. Since we did not further investigate this aspect specifically in this paper, I will expand the discussion on this topic in the next chapter, as part of more general considerations.

To achieve the maximal differentiation between resident microglia and infiltrating DCs, we performed parabiosis experiments joining the circulation of CD11c-eYFP mice with WT mice, and inducing brain ischaemia in the WT parabiont. In this way we could take advantage of the exclusive expression of eYFP by DCs infiltrating the injured brain from the periphery, and not by brain microglia.
In steady-state parabiont mice, we confirmed previous results indicating that DCs in the steady-state brain mainly locate in the leptomeningeal and choroid plexus compartment, rarely reaching the parenchyma (Bulloch et al., 2008). Our results however further highlight the fact that this population of DCs is a dynamic population that can traffic to/from the periphery, possibly sampling brain-related antigens even in steady-state conditions and participating in the maintenance of peripheral tolerance. Exactly how the pool of brain-patrolling DCs is maintained is currently unknown. In the skin and small intestine it has been recently proposed that a pool of pre-DC precursors may seed the tissue and give rise to cDCs, which themselves maintain proliferative capacity, and that upon infection the pool of tissue DCs is increased mainly by the infiltration of pre-DCs that differentiate in situ (Cabeza-Cabrerizo et al., 2019). However, the CNS is different, since resident parenchymal DCs have seldom been observed and the patrolling ones have been detected in “passing sites”, where it is difficult to discern between resident and trafficking cells.

In neuroinflammatory conditions, namely four days after inducing ischaemia in the WT mice of the parabiotic pairs, we observed increased numbers of eYFP$^+$ cells at the brain-CSF borders, and we demonstrated infiltration of eYFP$^+$ cells in the ischaemic parenchyma.
We also took advantage of this model to carry out a comparative transcriptomic profile of the infiltrating eYFP$^+$ cells versus the resident microglia extracted from ischaemic brains 4 days after stroke.
From the enormous amount of data generated by the RNA-Seq comparison, several observations could be made. Keeping in mind the methodological limitations, when comparing the transcriptome profile of our eYFP$^+$ infiltrating cells with the ones of populations deposited in the ImmGen consortium database, we could identify some similarities of our population with the ones reported in ImmGen, but we did not find complete correspondence. This is most probably due to the inflammatory situation of the ischaemic brain, which could be heavily skewing the gene expression of these cells. It is also possible that these cells may be showing brain-specific or at least brain-influenced characteristics as already proposed (Immig et al., 2015); a possibility that would be interesting to further investigate in animals in steady state. This could be possibly correlated to their crucial role in the patrolling of an immunologically privileged organ such as the brain, which is intrinsically potentially vulnerable and in need of strict immunosurvey.

When compared with microglia, eYFP$^+$ cells showed increased expression of several C-type lectin receptors, some of which, notably DNGR-1, Mincle and Dectin-1, have been implicated in immunomodulating functions (del Fresno et al., 2018; Martínez-López et al., 2019; unpublished data from our lab: Otxoa-de-Amezaga et al.). Microglia showed instead increased expression of TLR genes, overall suggesting at least some degree of cellular specialisation in the innate sensing capacity between the two populations analysed. As we observed in the other paper presented in this dissertation, the innate mechanisms that can be mediated by DCs in the ischaemic brain can have a strong impact on the pathology.

Interestingly, our RNA-Seq data, together with Ki67 staining data, suggested that microglia are more prone to proliferation than DCs (we also observed microglia proliferation in (Otxoa-de-Amezaga et al., 2019b)). This is in accordance with the observation that microglia cells mostly maintain themselves through CSF1R-dependent proliferation (Elmore et al., 2014), whereas brain DC populations depend more on the replacement granted by cells coming from the periphery (Anandasabapathy et al., 2011). We then also turned this characteristic of microglia to our advantage for the depletion experiments that we performed.

In parabiotic mice, we studied the identity of the infiltrating cells reaching the ischaemic brain from the periphery by flow cytometry phenotyping, with an antibody panel very similar to the one we developed for the first paper. In this way, we observed that the majority of DCs infiltrating the brain 4 days after ischaemia were CD172a$^+$ cDC2, with a smaller contribution by moDCs, cDC1 and pDCs. This is perfectly in line with the results obtained in the first paper presented, where cDC2 were the most prominent subpopulation of DCs throughout all the time points analysed.

Since we observed that the infiltrating DC population did not show signs of particularly active proliferation, we set out to investigate which signals were driving their recruitment to the ischaemic brain.
The RNA-Seq data from parabiotic mice indicated that DCs expressed higher levels of various chemokine receptors compared with microglia, and especially the expression of *Ccr1* and *Ccr2* mRNA was increased also compared with spleen DCs, indicating a possible role in the recruitment to the brain.
At the same time, we observed increased expression of several chemokine receptor ligands in the ischaemic brain, and we were able to identify microglia as the most prominent producer of *Ccl8* and *Ccl5*, both CCR1 ligands.
Eliminating microglia via CSF1R pharmacological inhibition, we observed how the infiltration of DCs was significantly reduced in microglia-depleted mice, uncovering a previously hypothesised (due to the known expression by microglia of various chemoattractant chemokines) but undescribed relationship.
This effect was directly attributable to the reduction of chemokine production in the ischaemic brain tissue, possibly not only by microglial cells, but also by other brain cells which could be indirectly influenced by the absence of microglia. Most importantly, the most prominently affected DC subpopulation were cDC1 cells.

All these observations on chemokines and their receptors put together led us to explore the possibility that CCR1 was involved in the infiltration of cDC1 cells to the ischaemic brain. By blocking CCR1 with a pharmacological antagonist, we observed that indeed the XCR1$^+$ cDC1 population was the only one who showed reduced infiltration 4 days after ischaemia. Most importantly, this led to a worsening of the neurological score in the animals that received the inhibitor, which was not visible in the control-treated animals, uncovering a possible beneficial role of cDC1 cells in the acute phase of stroke.
To further corroborate this observation, we analysed *Batf3*$^{-/-}$ mice, which constitutively lack cDC1 cells (Hildner et al., 2008), observing very similar results. In the absence of cDC1 cells, the mice had increased infarct volumes and worse

neurological outcome. These results were also confirmed in bone marrow chimeras where *Batf* knockout was present only in peripheral cells, indicating that indeed the beneficial effect is attributable to infiltrating cDC1 cells.

This effect of infiltrating cDC1 cells is quite surprising considering that their numbers in the ischaemic brain are significantly lower compared with cDC2. In our work, we did not further analyse the mechanism by which these cells may be mediating the beneficial effects we observed, but several pieces of evidence coming from other models may hint at their involvement in the regulation of the local innate immune response (del Fresno et al., 2018; Janela et al., 2019). Moreover, cDC1 have also been shown to maintain peripheral tolerance by cross-tolerising $CD8^+$ autoreactive T cells (Luckashenak et al., 2008), even though their absence did not overtly cause autoimmune reactions (MacNabb et al., 2019). Recently it has been demonstrated that in the skin this important tolerising function is dependent on MHCII-mediated interaction with Treg cells (Muth et al., 2012; Wohn et al., 2020). This collaboration between cDC1 and Treg cells is actually bidirectional, since in several experimental settings cDC1 have been observed to promote Treg cell differentiation and thus maintain tolerance (Arnold et al., 2019; Darrasse-Jèze et al., 2009; Khare et al., 2013).

It is plausible to hypothesise that these same mechanisms may be at work in the maintenance of peripheral tolerance to brain antigens, especially during brain ischaemia, where the involvement of Treg cells has been quite extensively demonstrated, and where they indeed seem to exert protective effects in the late phase of tissue recovery (Liesz et al., 2009).

The beneficial role attributed to cDC1 may be in fact dependent on their capacity to maintain tolerance to brain antigens through cross presentation, even though these mechanisms usually take place in secondary lymphoid tissues, not directly in the periphery (Joffre et al., 2012). Interfering with the infiltration of cells to the brain (via CCR1 antagonist) or eliminating completely cDC1 (with the *Batf3*$^{-/-}$) may be targeting different functions of cDC1, even if ultimately achieving similar results. Furthermore, it is important to keep in mind that the constitutive ablation of *Batf3* may be altering the physiological differentiation of T cells, especially Tregs (Lee et al., 2017).

Approaches targeted at interfering with specific interactions (e.g. with Tregs (Wohn et al., 2020)) or mechanisms may give more precise answers as to which of them are indeed most important in the observed brain protection.

All in all, whether adaptive tolerising mechanisms or innate modulation are at play by the cDC1 infiltrating the brain in the early phase of ischaemic stroke is an issue that requires further investigation.

GENERAL DISCUSSION

The role of DCs in brain physiology and pathology has been increasingly recognised, and has evolved together with the concept of immune privilege of the CNS (Galea et al., 2007).
Neuroinflammation has been traditionally more associated with resident microglia and infiltrating macrophages, so the study of the involvement of DCs in the immune response to neurological diseases is a relatively recent endeavour.
As already mentioned, I believe that one of the most relevant reasons for this lag in brain DC research is the technical difficulty related to their precise identification in the brain setting, due to the presence of cells that share common surface markers or that carry out similar functions, especially in neuroinflammatory states.
Both papers presented in this dissertation had to overcome this difficulty and resolved it in different ways, contributing to the knowledge about the role of DCs in the response to ischaemic stroke.

The two papers presented may be actually considered complementary: the first investigated the innate aspect of the DC-mediated regulation of the brain immune response to stroke, with emphasis on the role of cDC2; the second delved more in the differentiation of infiltrating DCs from resident microglia, their phenotypic characterisation, their capacity to elicit adaptive immune responses and the role of the cDC1 subpopulation.
Taken together, I believe they are a realistic (albeit limited) representation of the complexity of the injured brain DC population, based on the recent DC classification into subpopulations with distinctive characteristics and functions (Guilliams et al., 2016; Guilliams and van de Laar, 2015). We confirm the existence of this subdivision also in the ischaemic brain, where the specificity of markers, ontogeny and function appears to be maintained. However, the inflamed cerebral environment seems to confer some peculiar traits to brain DCs, which differ from DCs in other organs, as revealed by our transcriptomic analysis.

The RNA-Seq data we produced analysing infiltrating DCs will be useful as a reference for further subsequent studies on brain DCs, both in physiological and pathological conditions. Even though scientific research is increasingly moving towards single-cell approaches, these techniques are still not readily accessible, so these data may still be relevant for transcriptomic comparison at population level both for microglia and DCs.

Of course, given the observations of functional specificities of DCs subpopulations in the ischaemic brain, the molecular signatures of brain infiltrating subpopulations of DCs will require further investigation.

It is interesting and at the same time difficult to speculate about the significance of the contemporary infiltration into the brain of two different subpopulations of DCs with such diverse characteristics and ultimately different effects on stroke outcome. Despite only being a fraction of the number of cDC2, the infiltrating cDC1 cells seem to be able to exert beneficial functions, even though the underlying mechanisms are still to be unravelled. To me, this is particularly fascinating and leads me to contemplate the possibility that this infiltration, more than the cDC2 infiltration, is the most actively and strictly regulated, since it may be required as a safety mechanism mediated by peripheral tolerance induction.
Given their detrimental effect on the tissue, it is possible to speculate that the infiltration of cDC2 cells may be more a consequence of the inflammatory chemoattractant milieu of the brain resulting from the ischaemic injury, rather than an active recruitment of this cell type specifically. Nevertheless, we only analysed the effect of the presence of this cell type in a restricted early time window, and we mostly observed innate-like mechanisms at work. This limits the perspective on the possible role of both the subpopulations analysed in later stages of the disease, where they may be participating in the recovery and repair of the tissue, possibly more through antigen presentation-dependent mechanisms.

Another interesting topic of discussion that can be extrapolated from the results presented is the dichotomy between innate vs. adaptive functions of DCs in the context of brain ischaemia, or more generally of brain diseases.
Historically, the uptake of antigens in the periphery and their presentation to T cells in secondary lymph nodes to elicit an adaptive immune response has always been considered the main role of DCs in response to alterations of the homeostasis. Increasing evidence point at the possibility that DCs could act as mediators of the immune response independently from their direct involvement in the adaptive arm of immunity, through the engagement of PRR and the consequent release of cytokines (Clark et al., 2000; del Fresno et al., 2018; Durai and Murphy, 2016; Iborra et al., 2016; Martínez-López et al., 2019).
As pointed out in the first section of the discussion, we observed one such possible mechanism at work in the early phase of the response to ischaemic stroke by cDC2. Their rapid IL-23-mediated response, together with the antigen-independent activation capability of γδ T cells, leads to the jump start of a downstream cascade of events ultimately contributing to neuroinflammation.

Our results do not definitively exclude the involvement of some form of antigen presentation in this early response, but the combination of the rapidity and the cell types involved points more at an innate-like mechanism taking place on site.

The whole topic of antigen presentation of brain antigens after injury to the CNS is per se matter of debate. In stroke patients, an overt autoimmune response does not manifest, even if several hints have been observed: MBP-reactive T cells were found in the CSF and blood of stroke patients (Wang et al., 1992); antigen-specific autoreactive T cells were detected in the blood of stroke patients (Klehmet et al., 2016); brain antigens were found in secondary lymphoid tissue of patients (Planas et al., 2012); responses to various brain antigens by T cells and B cells have been observed (Mracsko et al., 2014; Ortega et al., 2015) and clonal expansion of T cells in experimental stroke has been detected (Liesz et al., 2013). In general, these observations were made at more delayed phases of stroke, starting from around day 7 after the onset of ischaemia. This makes it plausible to postulate an initial infiltration of naïve T cells, which carry out the detrimental effects observed in the early phases in an antigen-independent manner (Kleinschnitz et al., 2010), and then clonally expand thanks to the contact with APCs loaded with antigens, in the brain or in secondary lymphoid tissues. This makes sense also keeping in mind the accumulation of leukocytes and lymphocytes in the early phase of the stroke immune response (Gelderblom et al., 2009), which we have shown to exert deleterious interactions independent of antigen presentation.
The clonal expansion of brain-specific T cells may have a more relevant role in the delayed phase of stroke, from around day 7 after stroke onwards, when the regulation of the initially mostly pro-inflammatory environment needs to take place to give way to tissue repair (Liesz et al., 2009). The mechanisms of this late involvement are not clear, but there is evidence possibly excluding detrimental effects, since an increase in antigen-specific T cells did not worsen functional outcomes of experimental stroke on a long-term analysis (Römer et al., 2015).
In any case, it is important to understand these mechanisms, since the effects of the initial response may be carried over for long time. It has been shown that patients with severe stroke and patients that developed an infection in the first 15 days from admission were more prone to have a Th1-skewed T cell response to MBP 90 days after stroke (Becker et al., 2011). These consequences may be even more long lasting if we take into account the development of autoantibodies, that have been proposed to have a role in the development of post-stroke dementia (Doyle and Buckwalter, 2017).
Even if the papers presented in this dissertation did not further investigate the role of antigen presentation in stroke, our group recently showed that peripheral T cells

from stroke patients were able to respond to myelin peptide stimulation, confirming the presence of brain-primed T cells in the periphery. These cells, however, do not seem to be responsible for brain-directed autoimmune reactions, hinting at possible mechanisms of inhibition put in place to avoid autoimmunity (Miró-Mur et al., 2020).

The expression of immunomodulatory molecules that we observed in the infiltrating DCs may indeed be a local mechanism put in place to try to counteract the early infiltration of lymphocytes, or at least to drive them towards tolerance to brain antigens (mechanisms reviewed in Funes et al., 2019).

Furthermore, the immunosuppression that is detectable after stroke may be another, this time systemic, mechanism aimed at limiting overt autoimmune responses to the newly released stroke-derived brain antigens (Vogelgesang and Dressel, 2011). However, the possibility that patients develop infections as a consequence of this immunosuppression exists, and in this case the whole situation may change, facilitating a shift towards more evident autoimmune reactions (Becker et al., 2011; Becker et al., 2005).

It should be noted that the goal of both the physiological attempt at immunoregulation and the therapeutical strategies of immunomodulation is not the complete abolishment of the T cell response in the brain, since it has also been argued that antigen-specific Treg responses may be protective (Becker et al., 2003; Becker, 2009).

One of the possible therapeutic approaches that could stem from better knowledge of the involvement of DCs in the immune response of the ischaemic brain could be the use of tolerogenic DCs differentiated *in vitro* and loaded with brain-related antigens. This would be aimed at providing antigen specific immune tolerance, for example by inducing Treg differentiation or production of immunomodulatory molecules (Audiger et al., 2017).

The differences between the observations in patients and in experimental models may derive from the experimental approaches taken to investigate antigen presentation mechanisms. It is possible that the signs of effective T cell activation towards brain antigens observed in experimental models may be dependent on the frequent use of transgenic strains such as 2D2 or OTI and OTII, which make experimental observations easier, but possibly skew them in favour of activation because of the high affinity of these transgenic cells for their antigen (Keck et al., 2014). Furthermore, experimental antigens are possibly delivered in doses that do not necessarily reflect the natural availability of brain antigens, even after their release from the injured tissue.

Overall, in is evident that the response to stroke is complex, as is the involvement of the two main arms of immunity. As a consequence, the role of DCs, which act as bridge between the two, is equally intricate.
The presence of multiple DC subpopulations, the involvement of other putative APCs such as microglia, the overlapping infiltration dynamics and pleiotropic functions, all make for an extremely complicated setting to be able to isolate and attribute mechanisms and effects to specific cells.
This complexity gets transposed on the possible therapeutic approaches that could be developed upon the expansion of our knowledge of these phenomena, and it is possibly one of the causes of the current struggle to efficaciously translate from bench to bedside the brain protective therapies proposed for stroke.
Given our results, a DC-based therapy for stroke would need to be extremely specific. If given in the acute phase of stroke it would need to specifically block the deleterious effects mediated by cDC2 cells, without interfering with the beneficial role of cDC1.
It is possible that the currently available therapies target too broad populations of cells, which in reality may play different roles, complimentary or opposed, and may do so in different time frames. Ultimately, the goal would be to move not only research, but also treatment options towards a “single-cell medicine”, which uses extremely spatially and temporally targeted approaches.

Conclusions

Considering the objectives proposed for the present thesis and the results obtained during its development, the following conclusions can be drawn:

- Various subpopulations of DCs infiltrate the brain in the acute phase of ischaemia, being cDC2 the most prominent.

- Infiltrating cDC2 cells produce IL-23, which stimulates IL-17 production by γδ T cells, ultimately exerting a deleterious effect on stroke outcome.

- The disruption of the IL-23/IL-17 signalling axis reduces the production of *Cxcl1* by astrocytes and the consequent infiltration of neutrophils.

- Brain-infiltrating DCs display a differential transcriptomic signature when compared with resident microglia.

- Brain DCs are the most effective antigen-presenting cells in the ischaemic brain.

- Microglia attract DCs to the ischaemic brain via production of chemokines.

- cDC1 subpopulation is the most responsive to microglia chemoattraction and exerts beneficial functions in the ischaemic brain.

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