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Review

Homeostasis of Microglia in the Adult Brain: Review of Novel Microglia Depletion Systems

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Microglia are brain macrophages that emerge from early erythro-myeloid precursors in the embryonic yolk sac and migrate to the brain mesenchyme before the blood brain barrier is formed. They seed the brain, and proliferate until they have formed a grid-like distribution in the central nervous system that is maintained throughout lifespan. The mechanisms through which these embryonic-derived cells contribute to microglia homoeostasis at steady state and upon inflammation are still not entirely clear. Here we review recent studies that provided insight into the contribution of embryonically-derived microglia and of adult 'microglia-like' cells derived from monocytes during inflammation. We examine different microglia depletion models, and discuss the origin of their rapid repopulation after depletion and outline important areas of future research.

Trends

Microglia are unique tissue macrophages that exclusively derive from yolk sac progenitors without fetal monocyte contribution.

Microglia are long-lived cells with self-renewing capacity that persist inside the brain parenchyma from genesis until adulthood.

Microglia can be depleted efficiently by genetic and pharmacological tools, but repopulate within 5 days from brain-intrinsic precursors.

In an inflammatory context, dying or depleted microglia can be replaced by peripheral monocytes, which develop a microglia-like phenotype.

Microglia: A Unique Tissue Macrophage

Microglia are central nervous system (CNS)-resident myeloid cells that adopt various different functions. Robertson (1900) and Nissl (1898) were the first to report these cells, describing them as 'rod cells' ('*Staebchenzellen*' in German) because of their rod-like shape and noting their accumulation near inflammation-induced lesions in the CNS. Robertson was also the first to describe the phagocytic function of microglia: using Scharlach Red and hematoxylin staining, he was able to visualize fatty degeneration products that most likely resulted from destroyed neurons inside microglia. In 1919, Pio del Rio-Hortega, a Spanish scientist considered by many to be 'the father of microglia', provided an exceptional visualization of nervous tissue by inventing the silver carbonate method, naming the cells 'microglia', or the 'third element'. Del Rio-Hortega was not only capable of distinguishing microglia from oligodendrocytes or astrocytes; he also discovered their potential to change morphology from ramified to amoeboid under pathological conditions [1–3].

Microglia are the only immune cells present in the CNS parenchyma and are thus the first responders to environmental change. Under conditions of tissue damage such as that associated with bacterial or viral infections of the CNS, microglia play a critical role in clearing debris and restoring homeostasis in the CNS [4,5]. Microglia not only function as innate immune cells in pathological conditions, but also maintain tissue integrity in noninflammatory conditions [2,6–8]. It is clear that microglia are anything but 'resting' in steady state: as sensors they constantly extend and retract their processes to monitor the brain parenchyma for tissue damage or invaders [6,7]. In addition, emerging data reveal fundamental roles for microglia in the control of

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neuronal proliferation and differentiation as well as in the formation of synaptic connections (Stevens and McVicar, this issue). For example, microglia are involved in building up the neuronal network and are capable of phagocytizing malformed synapses [9].

Microglia form cellular grids within the CNS that are maintained through an organism's lifetime independently of circulating precursors. However, the factors critical for microglial maintenance under physiological conditions are not well defined. Moreover, the origin of 'new' microglia in inflammatory conditions associated with alterations of the microglial population or on depletion is unclear. Recent studies have suggested that microglial cells arise from internal pools rather than from bone marrow (BM) precursors [10,11] and that these cells can be distinguished from monocyte-derived cells even in the context of inflammation [12]. These studies provide important insights into microglial development and maintenance and describe how these populations respond to injury and inflammation. They both relied on microglial ablation systems with unique characteristics, advantages, and limitations in terms of specificity, toxicity, and efficacy that condition the experimental results. In the context of the current understanding of microglial natural ontogeny during development, we discuss the origin of microglia after experimental depletion, comparing different approaches of microglial ablation *in vivo*.

The Natural Development of Microglia

Defining the origin of microglia has long been a subject of debate. Del Rio-Hortega was the first to propose that microglia are of mesodermal origin and could possibly derive from meningeal macrophages infiltrating the brain during embryonic development [1]. Several studies described microglial precursors in the developing brain early during embryogenesis. Amoeboid microglia were detected as early as embryonic day E11.0 in the fetal mouse and rat brains [13–15]. Alliot *et al.* also described the appearance of microglia ($F4/80^+CD11b^+$ cells) in the brain rudiment even as early as E8.0 [16]. The identification of embryonic Mac-1 $(CD11b)^+$ cells as microglial progenitors in the developing brain was further supported by their high proliferative potential in a colony stimulating factor-1 (CSF-1, M-CSF)-dependent manner when plated on an astrocyte monolayer [17]. Concurrent with the establishment of embryonic circulation, in rat embryos macrophage-like cells were found in blood vessels and the embryonic mesenchyme from E10.5 [18]. Mizutani *et al.* detected $CX3CR1^+$ cells at E9.5 in the cephalic mesenchyme that subsequently infiltrated the neuroepithelium at E10.5 [19]. Interestingly, these aforementioned microglial progenitors in the developing brain phenotypically resembled yolk sac (YS) macrophages [16,19,20]. Together these studies described the early emergence of microglia in the developing brain that preceded the initiation of fetal liver and BM hematopoiesis and was concomitant with the appearance of YS hematopoiesis.

In the mammalian embryo, hematopoietic cells are generated at distinct sites in separate waves. The first wave is called primitive hematopoiesis and occurs in the blood islands of the extraembryonic YS at E7, giving rise to 'early' erythromyeloid precursors (EMPs) mainly forming primitive nucleated erythrocytes and primitive macrophages [21,22]. In a second wave at E8.25, 'late' c-Myb^+ EMPs emerge in the YS and these give rise locally to YS macrophages or subsequently migrate into the fetal liver through the blood circulation at E9.5 [22]. Concomitantly at E8.5, in the embryo proper in the para-aortic splanchnopleura (P-Sp) region, and later in the aorta, gonads, and mesonephros (AGM) regions, a new wave of hematopoietic progenitors emerges. These precursors then colonize the fetal liver and give rise to the definitive hematopoiesis that will eventually also generate adult hematopoietic stem cells in the BM. After E12.5, the fetal liver becomes the major hematopoietic organ during embryonic development [23].

Interestingly, in contrast to other macrophage populations in the adult or in the embryo, the development of YS macrophages is unique in that they bypass a monocyte stage [24]. YS

macrophages seed the embryonic tissues including the brain through the blood circulation, which develops gradually from E8.5 to E10 [25]. Notably at this early developmental stage, the generation of monocytes and other myeloid cells in the fetal liver has not commenced [26]. Thus, YS macrophages evidently constitute an independent lineage and arise before the development of other myeloid cells that differentiate from definitive hematopoietic stem cells. This is also reflected by the fact that the development of YS macrophages is independent of c-Myb, a transcription factor critical for the development of hematopoietic stem cells and their progeny, but relies on PU.1, a transcription factor crucial for other tissue macrophages [27].

That YS macrophages could be the progenitors of microglia was proposed as early as 1999. Pessac *et al.* described the presence of F4/80^{+}CD11b^{+} progenitors in the brain after their appearance in the YS [16]. Also, Alliot's work documented the presence of potential microglial progenitors in the YS and subsequently in the brain rudiment, with their numbers increasing considerably from E9.0/E9.5 until around 2 weeks after birth [16]. In agreement with these studies, in zebrafish embryos, macrophage-like cells appeared at an early stage in the YS before the emergence of other leukocytes and subsequently colonized the cephalic mesenchyme [28]. Only recently, with the advance of new fate-mapping models that allowed the tracing of YS progenitors, the unique ontogeny of microglia was definitely established [20,22,27,29–31]. Ginhoux *et al.* used a lineage-tracing model to label YS progenitors, including YS macrophages, by making use of runt-related transcription factor 1 (Runx1). Runx1 is a transcription factor critical for the emergence of hematopoietic stem cells. During early embryogenesis, Runx1 expression is restricted to the extra-embryonic YS thus allowing the tracing of YS progenitors using the tamoxifen (TAM)-inducible *Runx1-MER-Cre-MER* knock-in mice crossed to a reporter strain [3]. With this inducible system, they could show that adult microglia arise unequivocally from YS macrophages that seed the brain at E9.5, where they proliferate *in situ* and are maintained throughout adulthood [20]. Kierdorf *et al.* subsequently identified the precursors of the primitive YS macrophages/microglia. They found c-kit^{+} EMPs in the YS at E8 that differentiated into CD45^{+}c-kit^{lo}CX3CR1^{-} early precursors before their maturation and migration into the developing brain as CD45^{+}c-kit^{-}CX3CR1^{+} cells [31]. More recently, by using an *in utero* macrophage depletion system and by genetic tagging of YS and fetal liver hematopoietic cells, Hoeffel *et al.* extended the characterization of YS EMPs. Using again the Runx1 fate-mapping model and comparing the progenies of YS progenitors labeled by TAM at either E7.5 or E8.5, they identified two waves of EMPs that arise sequentially in the YS: an early wave of E7.5 EMPs that differentiate only locally in YS macrophages and a later wave of E8.5 EMPs that then either differentiate locally into YS macrophages or migrate to seed the fetal liver following the establishment of blood circulation from E9.0 [22]. However, this study clearly showed that most microglia arise from YS macrophages deriving from the early wave of EMPs.

It is important to note that microglia have a unique origin compared with other macrophages. Although YS macrophages seed all tissues at mid-gestation in the developing embryo, they are not essential for the generation of adult tissue-resident macrophages as they are later substituted by fetal monocytes that differentiate into tissue macrophages able to self-renew into adulthood [22]. The only exception are microglia. The reason for the absence of a fetal monocyte contribution to the microglial progenitor pool could lie in the lack of access to the developing brain. At around E13.5, when fetal monocytes start to colonize most embryonic tissues, there is only a negligible influx of fetal monocytes into the brain, which coincides with the formation of the blood–brain barrier (BBB) [32].

The unique ontogeny of microglia has been established in the steady state. Interestingly, however, after depletion of microglia either in the embryo or in the adult, microglia are replenished rapidly. The precursors for these new microglia are not yet fully characterized and whether

they fulfill the same characteristics as their embryonic counterparts in terms of CNS development, homeostasis, and functions in neuroinflammation is not entirely clear and is discussed below.

Consequences of Microglial Depletion during Embryogenesis

Under physiological conditions, as described above, embryonic microglia originate exclusively from YS macrophages. In mice lacking YS macrophages (e.g., *Csf1r*$^{-/-}$, *Pu.1*$^{-/-}$ mice), microglia progenitors are absent in the developing CNS [20,27]. Also, blocking antibody against CSF-1R (AFS98) administered at E6.5/E7.5 leads to rapid depletion of primitive macrophages in the YS, in all embryonic tissues, and of microglia in the developing CNS at E10.5–E14.5 [22,33]. Interestingly, while embryonic microglia were fully depleted from E10.5 to E14.5, only partial repopulation was observed at E17.5 and complete repopulation occurred only after birth [33]. This indicates that either a second wave of primitive hematopoiesis-independent precursors could give rise to microglia or a few remaining microglial progenitors could refill the empty niche. The nature of the repopulating cell is under investigation, but the recruitment of fetal monocytes at E14.5 into the brain of anti-CSF-1R antibody (AFS98)-treated embryos, a cell population normally absent in such tissue, was noted [22].

These observations suggest that fetal monocytes may give rise to microglia if they are able to access the brain rudiment. Whether such access to the brain rudiment is given by the absence of natural microglial progenitors provided after YS macrophage depletion or by the alteration of the BBB in these manipulated embryos remains to be clarified. Nevertheless, these observations are intriguing as they suggest that microglia may arise from two origins, although the functional consequences for brain homeostasis need to be investigated. We recently showed that perturbing normal embryonic microglial activity through depletion of its progenitors affects the progression of dopaminergic axons in the forebrain and the laminar positioning of subsets of neocortical interneurons [33]. Since defects in both dopamine innervation and cortical networks have been linked to neuropsychiatric diseases, these data provide new insights into how fetal microglial dysfunction can impact forebrain connectivity and reveal novel roles for embryonic microglia during normal assembly of brain circuits.

Tracking the Fate and Origin of Microglia during Inflammation

The concept of the mononuclear phagocyte system (MPS) stating that tissue macrophages are constantly replaced by circulating monocytes [34] has been revisited within the past few years. As observed in the case of microglia, it is becoming clear that most tissue-resident macrophage populations self-maintain locally in the steady state independently of circulating monocytes or other BM-derived precursors [23,35]. Nevertheless, in a BM transplantation setting associated with injuries caused by lethal irradiation, most tissue macrophages are reconstituted by donor-derived cells [36]. By contrast, microglia survive high-dose γ-irradiation and are not replaced by donor cells [10,20,37,38], in accordance with earlier seminal studies using BM chimeras [39]. Yet, several studies also using BM transplantation reported that BM-derived cells have the ability to migrate into the CNS and differentiate into parenchymal microglia-like cells [40,41]. However circulating myeloid cells can only enter the CNS if the BBB is damaged, such as when the CNS is inflamed or upon lethal irradiation [37,42]. For example, shielding the head of the mice during irradiation can prevent this monocytic influx efficiently [43]. To study the origin of microglial cells without harming the BBB by irradiation, the model of parabiosis can be used. Two parabionts, using for example a GFP reporter mouse as donor and a wild-type (WT) mouse as host, are surgically connected for several weeks. After this time, they share the same circulation, which leads to a blood chimerism of approximately 50%. By using such parabiotic mice, no GFP^{+} cells were found in the CNS of the WT mice, therefore demonstrating that monocytes do not contribute to the resident microglial pool [37]. Ajami *et al.* also combined parabiosis and myeloablation and showed that under neuroinflammatory conditions [experimental autoimmune

encephalomyelitis (EAE)], monocytes infiltrated the CNS and gave rise to 'microglia-like cells', but once inflammation was resolved monocytes did not persist inside the CNS and did not contribute to the long-term microglial pool [44].

Another important, but often overlooked, factor of such experimental models involving BM transplantation is the artificial introduction of a critical number of BM cells into the blood circulation. In conjunction with the inflammation of the BBB caused by irradiation, this creates a unique nonphysiological situation that is required for peripheral cells to differentiate into microglia-like cells [37].

Ablation of Microglia with Different Systems

Here we discuss the advantages and disadvantages of the various microglial ablation models and the origin of the 'new' repopulating microglia.

Genetic Deletion Models in Mice

Microglial ablation systems allow the study of their development and their role during CNS homeostasis and neurodegenerative and neuroinflammatory diseases. Genetic models of microglia-deficient mice carrying mutations in genes critical for their development such as PU.1, CSF-1R, and TGF-β [20,31,45] are not useful as these mice rarely survive into adulthood and have pleiotropic defects in organs other than the brain [46,47]. For example, in addition to the absent microglia, mice lacking CSF-1R exhibit severely reduced macrophage populations and display multiple developmental defects such as osteopetrosis. In another example, TGF-β1-deficient mice display drastic changes in the development of microglia [45] but also develop multiorgan autoimmune disease at an early age [48].

To circumvent such developmental defects, genetic tools and pharmacological inhibitors have been used to successfully deplete microglia. Importantly, although true depletion of microglia can be achieved with various depletion schemes, depletion is transient as microglia are always rapidly reconstituted following the depletion treatment.

Pharmacological Treatment for Microglial Ablation

Pharmacological treatment of mice with a reagent that inhibits CSF-1R signaling (PLX3397) resulted in ~99% ablation of microglia. Nevertheless, stopping the treatment resulted in fast repopulation of microglia within 5 days [10]. This model was used to study the source of the repopulating microglia. Two days after ceasing the treatment, it was found that CNS-intrinsic microglial precursors expressed transiently the neuronal marker nestin and gave rise to new microglia in the brain parenchyma (Box 1). In contrast to genetic tools, the advantage of this method is that the drug can be administered in the chow and can therefore be used to treat any mouse strain. Furthermore, the BBB remains intact after treatment, as assessed by Evans Blue straining [10]. However, this inhibitor is not specific for CSF-1R, as it also inhibits three other kinases: FLT3, PDGFR, and KIT [49,50]. In addition, systemic treatment with CSF-1R inhibitor leads to broad myelosuppression, impacting macrophages, HSCs, osteoclasts, and mast cells [51]. Potential peripheral microglial progenitors (like $Ly6C^+$ and $CX3CR1^+$ cells) in the BM and blood would be similarly affected.

Conditional Genetic Deletion Models

In 2005, a mouse line that allows the expression of a suicide gene, thymidine kinase (TK), under the myeloid-specific promoter CD11b [52] was generated. Treatment of these CD11b-HSVTK mice with ganciclovir (GCV) results in rapid elimination of $CD11b^+$ myeloid cells, including microglia. As elimination of all CD11b-expressing cells led to rapid mortality of the animals, the authors used a BM chimera strategy where BM from WT mice was grafted into irradiated CD11b-HSVTK host mice. Under these conditions, it was possible to eliminate only

Box 1. Is There a Predetermined Precursor of Microglia in the CNS?

For a long time it was believed that the brain had limited capacity for regeneration and tissue repair. This remains true for most of the CNS cell types, but some studies challenged this view by showing that many types of glial cells are able to self-maintain locally. Not only can oligodendrocyte progenitors establish their own homeostatic proliferation after depletion [67,68], but microglia can be stimulated to proliferate after pharmacological [10] or genetic [11] ablation. Both studies showed with immunohistological methods that the repopulating cells were $BrdU^+$ and $Ki67^+$. Elmore *et al.* (2013) showed that cell repopulation occurs fast and that the microglia express nestin, which is a marker for neural stem cells, although it can be expressed on macrophages as described below. These results suggested the existence of a microglial progenitor within the CNS, since their model resulted in >99% microglial depletion. They compared the *in vivo* situation with the *in vitro* generation of microglia using embryonic stem cells (ESCs) that first develop into neurons and after taking away the neuronal growth factors they develop into microglia [69]. Although having similar results, Bruttger *et al.* (2015) claimed that the entire microglial compartment is rebuilt from the 5–10% remaining microglial cells after ablation. Also here the intrinsic repopulated microglia transiently express nestin. Additionally, the BM-derived microglia that repopulate the brain after irradiation and depletion also express the nestin protein. Nestin expression in microglia could be shown, for example, in early stages of optic nerve injury [70] and traumatic brain injury [71], which indicates that nestin may be required for proliferation rather than serving as a stem cell marker. Importantly, as the study by Bruttger *et al.* (2013) used the promoter of CX_3CR1 as a cell fate-mapping system, they show that all repopulating cells are derived from CX_3CR1 expressing progenitor cells. The question of a certain predetermined progenitor cell within the brain is not yet finally resolved and needs to be further investigated.

irradiation-resistant $CD11b^+$ cells, most of which are microglia. Interestingly, microglia did not die after GCV treatment but instead displayed a 'freezing' phenotype in which microglial activation was blocked. The reason for this is most likely the toxic triphosphate, a product of GCV that selectively kills proliferating cells but not 'resting' ones. The authors then used the mouse model of multiple sclerosis, EAE, which can be induced by injection with a peptide derived from a myelin protein. On induction of EAE, the disease onset was delayed and clinical score was less severe in these mice. This suggests that microglia exert a proinflammatory function and contribute to the pathogenesis of EAE.

The CD11b-HSVTK system has been further developed by direct administration of GCV intracerebroventricularly (i.c.v.). After 1 week of i.c.v. GCV treatment, absolute numbers of $Iba\text{-}1^+$ cells are reduced by 40% and after 2 weeks 90% of microglia are depleted. The depletion efficiency even rises to more than 95% after 4 weeks of treatment, but it also leads to death of the mice possibly due to the myelotoxicity of the GCV (Table 1). The microglial compartment is repopulated within 2 weeks by peripheral $CCR2^+$ cells, most likely $Ly6C^{hi}$ monocytes, after treatment termination (Figure 1, Key Figure). Importantly, the i.c.v. treatment compromises the BBB locally, enabling peripheral cells to enter the brain parenchyma. Concomitant with the ablation, temporary astrocyte activation was reported, but neurons remained unchanged in terms of number and morphology [53].

For better *in vivo* manipulation of microglia, two independent laboratories generated Cx_3Cr1^{creER} animals. CX_3CR1 is a chemokine receptor, also known as the fractalkine receptor, highly expressed by microglia. The insertion sites in both targeting strategies were chosen according to previous studies in which the CX_3CR1 coding region was replaced with enhanced GFP [54]. Yona and colleagues introduced a Cre^{ER} cassette into the Cx_3Cr1 locus, whereas Parkhurst *et al.* (2013) generated a slightly different version of these animals, inserting a Cre^{ER}-IRES-eYFP cassette into the Cx_3Cr1 locus. A specific and long-lasting recombination in microglia was shown in Cx_3Cr1^{CreER} reporter mice [29,55]. Both microglia-specific Cre lines were crossed to the iDTR animals [11,56]. On systemic DT injections in Cx_3Cr1^{creER}:iDTR mice, microglia can be depleted with an efficiency of >90% [11,56]. This ablation is followed by fast repopulation within 5 days (Figure 1), similar to what was observed with the CSF-1R inhibition. Most likely, the new microglial compartment is built by those microglia that escaped ablation (remaining 5–10%). Massive proliferation in clusters was shown by BrdU incorporation [11]. Interestingly, if microglial depletion with the $CX3CR1^{CreER}$:iDTR system is combined with the BM-chimeric model, the new repopulating microglia originate from donor-derived cells [11], likely due to cells that infiltrate the

Table 1. Overview of Different Depletion Systems.

Depletion System	Treatment	Efficiency	Time Point of Depletion	Specificity	Side Effects	Related Publications
$CX_3CR1cre^{ER} \times iDTR^{ff}$	TAM + DTx	90%	6–8 weeks	Microglia only	Astrogliosis, cytokine storm	Bruttger *et al.* 2015 (in press)
$CX_3CR1cre^{ER} \times iDTR^{ff}$	TAM + DTx	95%	27–30 days	Microglia only	Impaired learning, dendritic spine	Parkhurst *et al.* 2013
CSF-1R blockade	PLX3397	99%	8 weeks (and 6 months)	Myelosuppression, HSCs	Astrogliosis	Elmore *et al.* 2014, *Neuron*
CSF-1R depletion of YS macrophages	AFS98 (anti-CSF-1R *in utero*)	99%	E6.5 and E7.5	YS macrophages	Dysregulation of wiring forebrain circuits, dopaminergic axons (forebrain) and interneurons (neocortex) affected	Squarzoni 2014, *Cell Reports* and Hoeffel 2015, *Immunity*
$CSF1R^{-/-}$	Genetic targeting	98%	Early embryonic development	Microglia, $Ly6C^{lo}$ monocytes, macrophages reduced	Lethal	Ginhoux *et al.* 2010
$PU.1^{-/-}$	Genetic targeting	98%	Early embryonic development	Microglia, lack mature myeloid cells and B cells	Lethal	Kierdorf *et al.* 2013
CNS $TGF\text{-}\beta^{-/-}$	Genetic targeting	80%	Early embryonic development	CNS specific	Motor abnormalities	Butovsky *et al.* 2013
CD11b-HSVTK	Irradiation + GCV pumps	80%	8 weeks	All $CD11b^{+}$ cells	BBB damage, myelotoxicity	Heppner *et al.* 2005/2010

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Key Figure

A View on the Three Major Systems to Ablate Microglia in Adult Mice

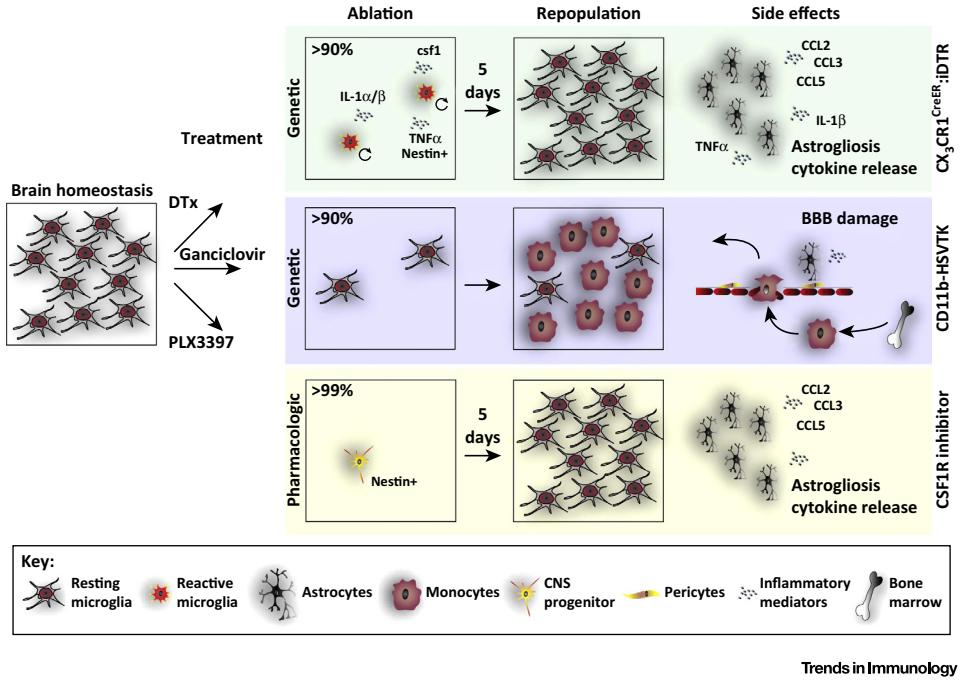

Figure 1. There are three well-studied ablation systems: (1) CX_3CR1^{CreER}:iDTR system (green) (Bruttger *et al.* 2015); (2) CD11b HSVTK system (blue) (Heppner *et al.* 2005); and (3) pharmacological treatment with PLX3397 (yellow) (Elmore *et al.* 2014). In the CX_3CR1^{CreER}:iDTR system, the microglia-specific Cre-recombinase drives the expression of diphtheria toxin receptor on the cell surface. On tamoxifen administration followed by systemic diphtheria toxin (DTx) injection, microglia undergo apoptosis and can be depleted with 90% efficiency. After 5 days, all microglia are repopulated by proliferating, non-depleted microglia. In the CD11b HSVTK system, all $CD11b^+$ cells can be depleted on ganciclovir treatment. To target microglia specifically one needs to do bone marrow reconstitution with wild-type BM, since it would be lethal to kill all $CD11b^+$ cells. The microglia are substituted by peripheral monocytes from the bone marrow. The pharmacological treatment of PLX3397, a CSF-1R inhibitor, wipes out >99% of microglia. This study suggests that $nestin^+$ progenitor cells within the CNS replace the microglial compartment. Ablation systems 1 and 3 are followed by fast repopulation of microglia from brain-intrinsic pools, accompanied by astrogliosis and an increase in proinflammatory cytokines. In ablation system 2, the cells that repopulate the brain have a peripheral origin. The blood–brain barrier (BBB) is compromised due to irradiation, which facilitates the entry of peripheral cells.

CNS already at the stage of BM transplantation, when the BBB is compromised due to irradiation.

The use of these TAM-inducible Cre lines allows microglia-specific targeting. Although CX_3CR1 is also expressed by other myeloid cells and precursors in the BM [57], Cre expression is lost over time after TAM treatment in the short-lived CX_3CR1^+ myeloid cells in contrast to the long-lived and self-renewing microglia [29,55]. Yet deficits in the peripheral myeloid compartment could affect the CNS [58]; therefore, a precise functional analysis of microglia needs to be conducted with caution, particularly when using knockout mice that affect both peripheral and CNS myeloid cells. Using the iDTR mice in another model to deplete oligodendrocytes, we were able to show that DTx can pass through the BBB [59–61]. The advantage of this new mouse model is that DTx can be injected intraperitoneally (i.p.) and there is no need for preparation of BM chimeras, resulting in very specific and effective microglial ablation. The disadvantage of this

system is that it requires the use of TAM. Treatment of mice with TAM may lead to an immunomodulatory phenotype as shown by the suppression of EAE [62], but our previous studies showed no such direct effect [63]. In addition, recent reports described an unexpected biological activity of the DTR in mice on inducible expression on conventional dendritic cells [64].

An important difference between the various models used for microglial ablation is the tissue response following depletion of cells. In one study [56], no change in CNS homeostasis in response to ablation was reported, whereas two other studies [10,11] have described clear upregulation in the expression of the astrocytic markers GFAP and S100, indicating that the mice exhibit astrogliosis. Furthermore, the expression of proinflammatory chemokines such as CCL2, CCL3, and CCL5 and cytokines such as TNFα and IL-1β were highly elevated following microglial depletion (Figure 1), suggesting the occurrence of a cytokine storm and sterile inflammation. This cytokine storm was, however, not reported by Parkhurst *et al.* [56].

One final important difference can be seen in behavioral changes following microglial ablation. CSF-1R inhibition [10] did not affect brain volume, cognition, or motor function as measured using an elevated plus maze, an open field, a Barnes maze, a rotarod, or contextual fear conditioning. By contrast, Parkhurst *et al.* describe deficits in motoric learning as a result of reduced dendritic spine formation and elimination. This was due to a change in the composition of NMDA and AMPA receptors, which leads to altered biochemical and electrophysiological properties of synapses. The different readouts for the behavioral tests, or even the different environments that influence the microbiota of these mice, might explain the different results [65].

Concluding Remarks

The development of new fate-mapping models helped in elucidating the ontogeny of microglia during embryonic development. However, the mechanisms by which these unique cells are maintained in the adult CNS remain unclear (see Outstanding Questions). We and others found that microglial depletion is followed by rapid repopulation. Furthermore, using two separate studies involving either pharmacological or genetic ablation of microglia, it was shown that microglia repopulate from brain-intrinsic sources. Whereas the existence of a certain predetermined microglial progenitor within the CNS is not yet completely proven (Box 1), both studies provide evidence for microglia displaying a very high proliferative capacity after depletion until normal microglial numbers are reestablished. The trigger for the rapid repopulation is not yet clear.

The rapid microglial repopulation following their depletion suggests the requirement of these cells by the CNS. Yet what will be the consequence if microglia are absent for longer periods of time? The different models reviewed here displayed rather short-term removal of microglia in the CNS. A hint regarding the long-term impact on the CNS of microglial deletion, even if the deletion is for a maximum of 14 days, arises from the Parkhurst work [56]. They found that mice lacking microglia suffer from cognitive dysfunctions, a phenotype that in our opinion might have been related to a cytokine storm and astrocyte activation and might not stem from the lack of microglia *per se*. In the aforementioned study it was however not addressed when the microglia were repopulated. Thus CNS integrity and function under conditions of long-term removal of microglia, or of genes expressed by microglia respectively, still need to be investigated, which may also help to clarify the exact functions of microglia under homeostatic conditions. This may be one of the most important topics in the analysis of mice depleted of microglia and in microglia-specific targeting.

Studies involving specific targeting of microglia with the CX_3CR1^{creER} mice already included functional assays. The results have shown that deletion of specific genes in microglia (e.g., TAK1, BDNF) can lead to reduced autoimmune CNS inflammation [29,45] and impaired dendritic spine

Outstanding Questions

What are the exact microglial functions in adult mice during steady state?

Is there a predetermined precursor of microglia in the CNS?

Can BM-derived microglia acquire a 'real' microglial phenotype over time?

Normal steady-state microglia are influenced by changes in microbiota. Can environmental factors influence the gene expression profile of BM-derived microglia within the brain (and after depletion or irradiation)?

Can BM-derived microglia fulfill complete microglial functionality?

formation [56]. In addition, when the *Mecp2* gene is rescued with CX_3CR1^{creER} in $Mecp2^{-/-}$ mice it was shown that the severe whole-body disease Rett syndrome was ameliorated [66], suggesting an important role for microglia and macrophages in disease regulation and implicating these cells as therapeutic targets in Rett syndrome. Altogether, the novel microglia-specific CX_3CR1^{creER} mouse strains represent an important new tool for the specific targeting of microglia. In addition to exploring microglial functions in an inflammatory context, the CX_3CR1^{creER} model makes it possible to study microglia in the steady state to gain a better understanding of the many different roles of these unique myeloid cells in the CNS.

The cells replacing microglia after depletion are derived from CNS-intrinsic precursors, but under certain conditions BM precursors can also give rise to microglia. These new microglia are able to 'masquerade' as 'original' microglia, exhibit a ramified shape with long processes, and are indistinguishable from intrinsic microglial cells by morphology. However, their genetic profile differs from that of YS-derived microglia and they cannot fully restore the microglial compartment over time, although BM-derived microglia reside in the brain for at least 12 weeks [11]. What remains an open question is whether, over a longer period of time, these newly engrafted microglia acquire the full characteristics of embryonically derived microglia, including function and self-maintenance, or whether, in the long run, CNS-resident microglia, or precursors of microglia yet to be discovered, will outnumber the transplanted cells. Indeed, one could speculate whether BM precursors could be utilized in the future for gene therapy in diseases in which microglia are dysfunctional. One could consider replacing genetically damaged microglia with BM precursors after a short period of eradication of the faulty cells. Another approach in microglial replacement and maturation could be to change environmental factors in the brain. Recently it was shown that microglial maturation and activation during health and disease strongly depend on the composition of microbiota [65], which would be potentially accessible for the treatment of microglia-mediated inflammatory or neuropsychiatric diseases.

The understanding of microglia and their embryonic development has been clarified nicely throughout the past 5 years. Recent developments in murine models allowing genetic manipulation of microglia and their fate mapping greatly helped to understand the unique nature of these cells and at the same time pioneered the way for the discovery of many more microglial functions.

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References

1. Del Rio-Hortega, P. (1932) Microglia. *Cytol. Cell. Pathol. Nerv. Syst* 2, 483–534
2. Kettenmann, H. *et al.* (2011) Physiology of microglia. *Physiol. Rev.* 91, 461–553
3. Samokhvalov, I.M. *et al.* (2007) Cell tracing shows the contribution of the yolk sac to adult haematopoiesis. *Nature* 446, 1056–1061
4. Koizumi, S. *et al.* (2007) UDP acting at P2Y6 receptors is a mediator of microglial phagocytosis. *Nature* 446, 1091–1095
5. Sierra, A. *et al.* (2010) Microglia shape adult hippocampal neurogenesis through apoptosis-coupled phagocytosis. *Cell Stem Cell* 7, 483–495
6. Davalos, D. *et al.* (2005) ATP mediates rapid microglial response to local brain injury *in vivo*. *Nat. Neurosci.* 8, 752–758
7. Nimmerjahn, A. *et al.* (2005) Resting microglial cells are highly dynamic surveillants of brain parenchyma *in vivo*. *Science* 308, 1314–1318
8. Prinz, M. and Priller, J. (2014) Microglia and brain macrophages in the molecular age: from origin to neuropsychiatric disease. *Nat. Rev. Neurosci.* 15, 300–312
9. Paolicelli, R.C. *et al.* (2011) Synaptic pruning by microglia is necessary for normal brain development. *Science* 333, 1456–1458
10. Elmore, M.R. *et al.* (2014) Colony-stimulating factor 1 receptor signaling is necessary for microglia viability, unmasking a microglia progenitor cell in the adult brain. *Neuron* 82, 380–397
11. Bruttger, J.K. *et al.* (2015) Genetic cell ablation reveals clusters of local self- renewing microglia in the mammalian central nervous system. *Immunity* 43, 92–106
12. Yamasaki, R. *et al.* (2014) Differential roles of microglia and monocytes in the inflamed central nervous system. *J. Exp. Med.* 211, 1533–1549
13. Ashwell, K. (1990) Microglia and cell death in the developing mouse cerebellum. *Brain Res. Dev. Brain Res.* 55, 219–230

14. Ashwell, K. (1991) The distribution of microglia and cell death in the fetal rat forebrain. *Brain Res. Dev. Brain Res.* 58, 1–12
15. Perry, V.H. *et al.* (1985) Immunohistochemical localization of macrophages and microglia in the adult and developing mouse-brain. *Neuroscience* 15, 313–326
16. Alliot, F. *et al.* (1999) Microglia derive from progenitors, originating from the yolk sac, and which proliferate in the brain. *Brain Res. Dev. Brain Res.* 117, 145–152
17. Alliot, F. *et al.* (1991) Microglial progenitors with a high proliferative potential in the embryonic and adult mouse brain. *Proc. Natl. Acad. Sci. U.S.A.* 88, 1541–1545
18. Sorokin, S.P. *et al.* (1992) Macrophage development: II. Early ontogeny of macrophage populations in brain, liver, and lungs of rat embryos as revealed by a lectin marker. *Anat. Rec.* 232, 527–550
19. Mizutani, M. *et al.* (2012) The fractalkine receptor but not CCR2 is present on microglia from embryonic development throughout adulthood. *J. Immunol.* 188, 29–36
20. Ginhoux, F. *et al.* (2010) Fate mapping analysis reveals that adult microglia derive from primitive macrophages. *Science* 330, 841–845
21. Orkin, S.H. and Zon, L.I. (2008) Hematopoiesis: an evolving paradigm for stem cell biology. *Cell* 132, 631–644
22. Hoeffel, G. *et al.* (2015) c-Myb^{+} erythro-myeloid progenitor-derived fetal monocytes give rise to adult tissue-resident macrophages. *Immunity* 42, 665–678
23. Ginhoux, F. and Jung, S. (2014) Monocytes and macrophages: developmental pathways and tissue homeostasis. *Nat. Rev. Immunol.* 14, 392–404
24. Takahashi, K. *et al.* (1989) Differentiation, maturation, and proliferation of macrophages in the mouse yolk sac: a light-microscopic, enzyme-cytochemical, immunohistochemical, and ultrastructural study. *J. Leukoc. Biol.* 45, 87–96
25. McGrath, K.E. *et al.* (2003) Circulation is established in a stepwise pattern in the mammalian embryo. *Blood* 101, 1669–1676
26. Naito, M. *et al.* (1990) Development, differentiation, and maturation of macrophages in the fetal mouse liver. *J. Leukoc. Biol.* 48, 27–37
27. Schulz, C. *et al.* (2012) A lineage of myeloid cells independent of Myb and hematopoietic stem cells. *Science* 336, 86–90
28. Herbomel, P. *et al.* (1999) Ontogeny and behaviour of early macrophages in the zebrafish embryo. *Development* 126, 3735–3745
29. Goldmann, T. *et al.* (2013) A new type of microglia gene targeting shows TAK1 to be pivotal in CNS autoimmune inflammation. *Nat. Neurosci.* 16, 1618–1626
30. Hoeffel, G. *et al.* (2012) Adult Langerhans cells derive predominantly from embryonic fetal liver monocytes with a minor contribution of yolk sac-derived macrophages. *J. Exp. Med.* 209, 1167–1181
31. Kierdorf, K. *et al.* (2013) Microglia emerge from erythromyeloid precursors via Pu.1- and Irf8-dependent pathways. *Nat. Neurosci.* 16, 273–280
32. Daneman, R. *et al.* (2010) Pericytes are required for blood–brain barrier integrity during embryogenesis. *Nature* 468, 562–566
33. Squarzoni, P. *et al.* (2014) Microglia modulate wiring of the embryonic forebrain. *Cell Rep.* 8, 1271–1279
34. van Furth, R. *et al.* (1972) The mononuclear phagocyte system: a new classification of macrophages, monocytes, and their precursor cells. *Bull. World Health Organ.* 46, 845–852
35. Epelman, S. *et al.* (2014) Origin and functions of tissue macrophages. *Immunity* 41, 21–35
36. Hashimoto, D. *et al.* (2013) Tissue-resident macrophages self-maintain locally throughout adult life with minimal contribution from circulating monocytes. *Immunity* 38, 792–804
37. Ajami, B. *et al.* (2007) Local self-renewal can sustain CNS microglia maintenance and function throughout adult life. *Nat. Neurosci.* 10, 1538–1543
38. Greter, M. *et al.* (2005) Dendritic cells permit immune invasion of the CNS in an animal model of multiple sclerosis. *Nat. Med.* 11, 328–334
39. Hickey, W.F. and Kimura, H. (1988) Perivascular microglial cells of the CNS are bone-marrow derived and present antigen *in vivo*. *Science* 239, 290–292
40. Brazelton, T.R. *et al.* (2000) From marrow to brain: expression of neuronal phenotypes in adult mice. *Science* 290, 1775–1779
41. Eglitis, M.A. and Mezey, E. (1997) Hematopoietic cells differentiate into both microglia and macroglia in the brains of adult mice. *Proc. Natl. Acad. Sci. U.S.A.* 94, 4080–4085
42. Mildner, A. *et al.* (2009) CCR2^{+}Ly-6C^{hi} monocytes are crucial for the effector phase of autoimmunity in the central nervous system. *Brain* 132, 2487–2500
43. Mildner, A. *et al.* (2007) Microglia in the adult brain arise from Ly-6C^{hi}CCR2^{+} monocytes only under defined host conditions. *Nat. Neurosci.* 10, 1544–1553
44. Ajami, B. *et al.* (2011) Infiltrating monocytes trigger EAE progression, but do not contribute to the resident microglia pool. *Nat. Neurosci.* 14, 1142–1149
45. Butovsky, O. *et al.* (2014) Identification of a unique TGF-β-dependent molecular and functional signature in microglia. *Nat. Neurosci.* 17, 131–143
46. Scott, E.W. *et al.* (1994) Requirement of transcription factor Pu.1 in the development of multiple hematopoietic lineages. *Science* 265, 1573–1577
47. McKercher, S.R. *et al.* (1996) Targeted disruption of the PU.1 gene results in multiple hematopoietic abnormalities. *EMBO J.* 15, 5647–5658
48. Shull, M.M. *et al.* (1992) Targeted disruption of the mouse transforming growth factor-beta-1 gene results in multifocal inflammatory disease. *Nature* 359, 693–699
49. Chitu, V. *et al.* (2012) PSTPIP2 deficiency in mice causes osteopenia and increased differentiation of multipotent myeloid precursors into osteoclasts. *Blood* 120, 3126–3135
50. Thompson, M.L. *et al.* (2015) Targeting cells of the myeloid lineage attenuates pain and disease progression in a prostate model of bone cancer. *Pain* 156, 1692–1702
51. Cornelis, S. *et al.* (2005) Apoptosis of hematopoietic cells induced by growth factor withdrawal is associated with caspase-9 mediated cleavage of Raf-1. *Oncogene* 24, 1552–1562
52. Heppner, F.L. *et al.* (2005) Experimental autoimmune encephalomyelitis repressed by microglial paralysis. *Nat. Med.* 11, 146–152
53. Grathwohl, S.A. *et al.* (2009) Formation and maintenance of Alzheimer's disease beta-amyloid plaques in the absence of microglia. *Nat. Neurosci.* 12, 1361–1363
54. Jung, S. *et al.* (2000) Analysis of fractalkine receptor CX_3CR1 function by targeted deletion and green fluorescent protein reporter gene insertion. *Mol. Cell. Biol.* 20, 4106–4114
55. Yona, S. *et al.* (2013) Fate mapping reveals origins and dynamics of monocytes and tissue macrophages under homeostasis. *Immunity* 38, 79–91
56. Parkhurst, C.N. *et al.* (2013) Microglia promote learning-dependent synapse formation through brain-derived neurotrophic factor. *Cell* 155, 1596–1609
57. Geissmann, F. *et al.* (2010) Development of monocytes, macrophages, and dendritic cells. *Science* 327, 656–661
58. Dantzer, R. *et al.* (2008) From inflammation to sickness and depression: when the immune system subjugates the brain. *Nat. Rev. Neurosci.* 9, 46–57
59. Locatelli, G. *et al.* (2012) Primary oligodendrocyte death does not elicit anti-CNS immunity. *Nat. Neurosci.* 15, 543–550
60. Buch, T. *et al.* (2005) A Cre-inducible diphtheria toxin receptor mediates cell lineage ablation after toxin administration. *Nat. Methods* 2, 419–426
61. Gritsch, S. *et al.* (2014) Oligodendrocyte ablation triggers central pain independently of innate or adaptive immune responses in mice. *Nat. Commun.* 5, 5472
62. Elloso, M.M. *et al.* (2005) Suppression of experimental autoimmune encephalomyelitis using estrogen receptor-selective ligands. *J. Endocrinol.* 185, 243–252
63. Yogev, N. *et al.* (2012) Dendritic cells ameliorate autoimmunity in the CNS by controlling the homeostasis of PD-1 receptor^{+} regulatory T cells. *Immunity* 37, 264–275

64. van Blijswijk, J. *et al.* (2015) Altered lymph node composition in diphtheria toxin receptor-based mouse models to ablate dendritic cells. *J. Immunol.* 194, 307–315

65. Erny, D.H.D.A. *et al.* (2015) Host microbiota constantly control maturation and function of microglia in the CNS. *Nat. Neurosci.* 18, 965–977

66. Cronk, J.C. *et al.* (2015) Methyl-CpG binding protein 2 regulates microglia and macrophage gene expression in response to inflammatory stimuli. *Immunity* 42, 679–691

67. Hughes, E.G. *et al.* (2013) Oligodendrocyte progenitors balance growth with self-repulsion to achieve homeostasis in the adult brain. *Nat. Neurosci.* 16, 668–676

68. Robins, S.C. *et al.* (2013) Extensive regenerative plasticity among adult NG2-glia populations is exclusively based on self-renewal. *Glia* 61, 1735–1747

69. Beutner, C. *et al.* (2010) Generation of microglial cells from mouse embryonic stem cells. *Nat. Protoc.* 5, 1481–1494

70. Wohl, S.G. *et al.* (2011) *In situ* dividing and phagocytosing retinal microglia express nestin, vimentin, and NG2 *in vivo*. *PLoS ONE* 6, e22408

71. Kaya, S.S. *et al.* (1999) Expression of nestin after traumatic brain injury in rat brain. *Brain Res.* 840, 153–157