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Opinion

To Kill a Microglia: A Case for CSF1R Inhibitors

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Microglia, the brain's immune sentinels, have garnered much attention in recent years. Researchers have begun to identify the manifold roles that these cells play in the central nervous system (CNS), and this work has been greatly facilitated by microglial depletion paradigms. The varying degrees of spatiotemporal manipulation afforded by such techniques allow microglial ablation before, during, and/or following insult, injury, or disease. We review the major methods of microglial depletion, including toxin-based, genetic, and pharmacological approaches, which differ in key factors including depletion onset, duration, and off-target effects. We conclude that pharmacological CSF1R inhibitors afford the most extensive versatility in manipulating microglia, making them ideal candidates for future studies investigating microglial function in health and disease.

Highlights

Microglial depletion animal models provide insight into microglial cell function in the healthy and diseased brain.

Microglia are dependent on colony-stimulating factor 1 receptor (CSF1R) signaling for survival in the vertebrate brain.

CSF1R inhibition allows rapid and titratable myeloid cell population manipulation in the rodent CNS.

We posit that optimal microglial depletion models (e.g., CSF1R inhibitors) should ideally enable the elimination of these cells for any duration of time in different cell subsets. These models might also stimulate microglial repopulation in any model or species, and act in a clinically relevant fashion.

Manipulating Microglia for Insight into Brain Function: Tools at Hand

Microglia are the primary immune cells of the brain and, together with perivascular, **choroid plexus** (see Glossary), and **meningeal macrophages**, comprise the macrophage compartment of the CNS. Under steady-state conditions, microglia are dynamic surveyors of the CNS, occupying distinct non-overlapping territories where they constantly extend and retract their processes to sample the local milieu and maintain tissue homeostasis [1–3]. Recent studies have shown that these cells are long-lived, rely on self-renewal for population maintenance, and exhibit brain region-dependent molecular and transcriptional heterogeneity [4–6]. During disease, aging, or injury, microglia undergo context-dependent transcriptional, morphological, and functional remodeling [7,8], the resolution of which is generally beneficial or crucial for recovery. Variants of microglia-associated genes have also been identified as risk factors for Alzheimer's disease (AD) [9,10], frontotemporal dementia (FTD) [11], Parkinson's disease (PD) [12], and amyotrophic lateral sclerosis (ALS) [13], among others, implicating these cells in the initiation and progression of these CNS disorders. Experimental approaches to deplete resident microglia have provided unprecedented insights into the roles that these cells play in the healthy, injured, and diseased mouse brain. Several methods have been developed that allow the microglial population to be manipulated using toxin-, pharmacology-, and genetics-based approaches. Each technique has its own advantages and limitations regarding factors such as the extent and duration of microglial depletion, treatment invasiveness, physiological side effects, species-specific effects, and other potential confounds, that should be discussed to correctly interpret the studies employing them. We argue here that inhibitors of the colony-stimulating factor 1 (CSF1) receptor (CSF1R) – a crucial receptor for microglial survival – may offer one of the most advantageous approaches for eliminating and studying microglia.

Toxin-Based Models of Microglial Depletion

Early approaches to deplete microglia involved the generation of *Cd11b–HSVTK* transgenic mice, which overexpress the herpes simplex virus-derived thymidine kinase (HSVTK) under the control of the *Cd11b* promoter (i.e., in cells of myeloid origin). In the presence of ganciclovir, HSVTK is activated and induces apoptosis in $CD11b^+$ mitotic cells [14,15]. However, systemic and extended administration of ganciclovir leads to fatal anemia owing to loss of $CD11b^+$ cells that are necessary for normal hematopoiesis [15] and enucleation of red blood cells [16], among

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other side effects [17]. To circumvent this myelotoxicity, investigators have relied on either wild-type (WT) bone marrow (BM) transfer [15] or intracerebroventricular (i.c.v.) administration of ganciclovir [18], which requires surgical implantation of an osmotic pump [19,20] and provides more selective treatment for microglial elimination.

An alternative genetic approach to deplete myeloid cell populations, including microglia, uses diphtheria toxin (DT)-based models. These combine myeloid promoter-driven Cre recombinase mouse lines with transgenic mice harboring genes for diphtheria toxin receptor (*DTR*) or diphtheria toxin A (*DTA*) downstream of *loxP*-flanked STOP sequences, which are removed upon Cre recombination to drive gene expression in myeloid cells [21]. Subsequent administration of DT causes acute cell death in myeloid cells expressing *DTR*, an effect that can be similarly achieved by direct cellular expression of *DTA* [21]. Studies generally utilize either *Cx3cr1*$^{cre/+}$ or *Cx3cr1*$^{creER/+}$ mouse lines to achieve constitutive or inducible DTR/DTA expression and cell depletion, respectively, although the availability of newer microglial Cre lines (e.g., *Tmem119*) may provide more specific interventions [22–24]. Similar DTR-based methods for myeloid tissue depletion, including microglia, have recently been extended to rat models [25]. Of relevance, inducible *Cx3cr1*$^{creER/+}$ animals depend upon tamoxifen administration for recombination and expression of DTR/DTA in CX3CR1^{+} cells, providing greater temporal control than constitutive Cre lines [23,26,27]. An additional advantage of this method includes the ability to selectively target microglia over circulating monocytes and other short-lived myeloid populations by exploiting the higher turnover rate of the latter [23].

However, DTR models only offer short-lived microglial depletion (<5 days [23]), induce a cytokine storm [23], rely on Cre recombinase which itself is toxic, and utilize tamoxifen (for inducible Cre lines) that binds to and is biologically active on macrophages and other estrogen receptor-expressing cells [1]. There are additional confounds related to the genetics involved, such as haploinsufficiency of the Cre driver gene, reported leakiness [28,29], and broad cell death of CX3CR1^{+} myeloid cell populations. The development of destabilized Cre recombinase (DD-Cre)-based depletion systems will be able to address some, but not all, of these issues (e.g., gene induction via the antibiotic trimethoprim instead of tamoxifen); nevertheless, these approaches also have their own unique technical challenges [30]. Alternatively, **clodronate liposomes** provide for a toxin-based method to target microglia and other macrophages without genetic modification by virtue of the liposome-encapsulated drug's specific uptake and lysosomal processing by phagocytic cells, thereby triggering apoptosis [31]. However, clodronate liposomes do not cross the blood–brain barrier (BBB), and thus require surgical infusion directly into the CNS in animal models, along with several other caveats (e.g., rapid but short-lived depletion, inflammatory cytokine induction), summarized in Table 1 (Key Table) [31–33].

Colony-Stimulating Factor 1 (CSF1) Receptor Signaling and the Evolution of Genetic Microglial Depletion Models

Extensive research over the past 50 years has demonstrated that signaling through CSF1 and its cognate receptor (CSF1R) is essential for microglial cell survival, and this pathway has played a fundamental role in the development of genetic models of microglial depletion. CSF1, also known as macrophage colony-stimulating factor (M-CSF), is a hematopoietic growth factor/cytokine that is involved in promoting macrophage proliferation, differentiation, and survival [34]. The sole receptor for CSF1 (CSF1R) is a membrane-spanning tyrosine kinase receptor, the product of the *c-Fms* proto-oncogene [34]. *Csf1r* is expressed in, and mostly restricted to, macrophage and microglia populations, as evidenced by *in situ* hybridization [35] and *Csf1r* promoter-driven GFP expression in transgenic MacGreen mice [36,37]. The central role of CSF1/CSF1R in myeloid biology was discovered upon characterization of osteopetrotic *op/op*

Glossary

5×FAD mouse model: a transgenic mouse model of Alzheimer's disease (AD), in which mice express human amyloid precursor protein (APP) and presenilin 1 (PSEN1) transgenes harboring five familial AD (FAD)-causing mutations.
Agenesis of the corpus callosum: a rare brain disorder in which the corpus callosum, a white matter tract that connects the two brain hemispheres, is partially or completely absent.
Choroid plexus: a structure located in the ventricles that plays an important role in regulating immune cell entry into the CNS and producing cerebrospinal fluid.
Clodronate liposomes: clodronate-encapsulated liposomes are phagocytosed by macrophages and, upon engulfment, result in the release of clodronate into the phagocytic cell, effectively inducing apoptosis and macrophage ablation.
Complement: the complement system or cascade plays a major part in the innate immune system. Complement proteins are soluble plasma proteins that opsonize pathogens and other pathological targets for clearance, effectively serving as molecular danger or 'eat me' signals to phagocytes.
Contextual fear memory: subjects are exposed to a specific neutral condition or context (e.g., room, tone, or light) that is associated with an aversive stimulus (e.g., shock), resulting in a fear response to the neutral context. This model allows the study of contextual fear learning and memory. Refers to the ability of subjects to recall the context that predicts danger or a fearful stimulus.
GABAergic interneurons: neurons that produce γ-aminobutyric acid (GABA), the main inhibitory neurotransmitter of the adult brain.
Meningeal macrophages: the population of macrophages that reside in the meninges that cover and protect the brain and spinal cord.
Neurogenesis: the process by which new functional neurons are generated from adult neural precursor cells.
Oligodendrocyte: a distinct type of glial cell in the CNS that plays a central role in myelination to support and insulate neuronal axons.
Perineuronal nets: specialized extracellular matrix structures that surround some types of neuronal cell bodies and dendrites in the mammalian

mice (*Csf1*^{op}/*Csf1*^{op}), which harbor a spontaneous mutation in the *Csf1* gene with consequent loss of CSF1 [38,39]. Research on these mice revealed diverse phenotypic changes including osteoporosis, brain abnormalities (e.g., slowed neuronal process outgrowth, abnormal auditory and visual evoked potentials, aberrant cortical circuitry) [40], substantially reduced macrophage/monocyte populations in blood, bone, and BM [41,42], and significantly reduced microglial densities in white matter tracts, as well as modestly reduced microglial densities in the grey matter/cerebral cortex relative to WT mice [43].

For improved understanding of CSF1R signaling, *Csf1r*$^{-/-}$ mice were generated. These mice display an exacerbated version of the *Csf1*^{op}/*Csf1*^{op} phenotype, including skeletal deformities, shortened lifespan, and neurodevelopmental abnormalities [37,44]. Both *Csf1r*$^{-/-}$ and *Csf1*^{op}/*Csf1*^{op} mice exhibit reduced peripheral tissue-resident macrophage populations relative to WT mice; however, only partial brain region-dependent microglial depletion is evident in *Csf1*^{op}/*Csf1*^{op} mice, whereas *Csf1r*$^{-/-}$ mice display a brain-wide absence of microglia [37,45]. The discrepancy between *Csf1*^{op}/*Csf1*^{op} and *Csf1r*$^{-/-}$ microglial numbers was explained by the discovery of a second ligand for CSF1R, IL-34 [46], which when absent also results in substantial microglial reductions, for example in *Il34*$^{lacZ/lacZ}$ mice [47]. Despite similar biological activities, *Il34* and *Csf1* exhibit distinct spatial and temporal expression patterns [48,49] that differentially drive regional microglial identity and function [50]. *Il34* expression is detected in the embryonic brain at embryonic (E) day E11.5, earlier than *Csf1* expression at E13.5; although both ligands are broadly expressed in the adult, *Il34* expression at both postnatal (P) days P8 and P60 is significantly elevated in the cerebral cortex, olfactory bulb, striatum, and hippocampus relative to *Csf1* mRNA expression [49]. By contrast, a recent study showed that CSF1, independently of IL-34, also serves as an essential factor for human and mouse cerebellar microglia [50]. Microglial reductions and impaired survival have furthermore been observed in mice with disrupted expression of other microglia-associated gene products, including transcription factors *Spi1* (*Pu.1*) [51] and *Irf8* [52], as well as *Tgfb1* [53] (summarized in Table 1). However, conditional knockout mice harboring a loxP-flanked exon within the *Csf1r* gene (i.e., *Csf1r*$^{flox/flox}$) have allowed spatial and temporal control of microglia upon combination with the appropriate Cre lines [54]. For example, microglia-specific ablation of *Csf1r* can be achieved by driving Cre expression under a promoter for a microglia-specific transcription factor, for example *Sall1*$^{creER/+}$*Csf1r*$^{flox/flox}$ mice, resulting in transient elimination of microglia with tamoxifen [55].

Recently, a constitutive genetic model was generated, the *Csf1r*$^{\Delta FIRE/\Delta FIRE}$ mouse, in which a portion of intron 2 of *Csf1r* was knocked out – a region containing a highly conserved super-enhancing *Fms* intronic regulatory element (FIRE) that has binding sites for many macrophage transcription factors. Although completely lacking microglia, *Csf1r*$^{\Delta FIRE/\Delta FIRE}$ mice appear to be developmentally normal, healthy, and fertile [56], providing a novel model to explore microglial roles in both development and adulthood. Similarly, *Csf1r*$^{-/-}$ rats have recently been described which, unlike mice, survive into adulthood despite a dramatic loss of microglia, with little overt neurological phenotype other than reduced myelination (Table 1) [57]. A spectrum of autosomal dominant and recessive neurological skeletal disorders have been reported in humans with mutations in *Csf1r*, and, although outside the scope of this article, these cases warrant further investigation to answer crucial questions regarding CSF1R function, haploinsufficiency, convergence with animal models, and loss of CSF1R in different myeloid cell populations. Nevertheless, accumulating evidence indicates that disease severity in humans and animal models is dependent on residual CSF1R function [58]. Specifically, individuals homozygous for CSF1R loss-of-function mutations exhibit early lethality, complete absence of microglia, and several structural brain anomalies including **agenesis of the corpus callosum**, enlarged lateral and fourth ventricles, and impaired cerebellar development [59], recapitulating many of the phenotypes observed in

CNS to regulate synaptic plasticity and stabilization.

PSD95: a major component of excitatory synapses that acts as a crucial scaffolding protein localized at the postsynaptic density (PSD) of excitatory synapses to regulate many receptors, channels, and signaling molecules. PSD95 is commonly utilized as a marker for labeling postsynaptic synapses.

Synaptophysin: also known as major synaptic vesicle protein p38, synaptophysin is an integral membrane glycoprotein that is localized to presynaptic vesicles and ubiquitously expressed in all neurons. It is commonly utilized as a marker for labeling synaptic terminals.

Tauopathy: a class of neurodegenerative disease caused by or associated with the aggregation or misfolding of tau protein in the brain.

Key Table

Table 1. Common Models of Microglial Depletion[a]

Approach	Duration	Extent of depletion	Cytokine storm	Requires invasive surgery	Requires genetic crosses	Potential gene haploinsufficiency	Will affect peripheral cells	Dependent on exogenous agent	Developmental defects	Species	Refs
Advantages						**Disadvantages**					
CSF1R inhibitors	Indefinite	0–100%	No	No	No	No	Yes[b]	Yes	No[c]	Any	[62,63,65, 75,77,98]
• No cognitive/behavioral impairments. • No BBB damage. • Orally bioavailable. • FDA approved. • Elimination sustainable virtually indefinitely. • Inhibitor cessation induces cell repopulation.						• Potential off–target effects of PLX3397 on related tyrosine kinase receptors (e.g., c–Kit, FLT3). • Not suitable for some developmental studies due to potential neuronal and/or osteoclast CSF1R expression.					
Diphtheria toxin–mediated	<5 days	80–90%	Yes	No	Yes	Yes	Yes[d]	Yes	No[c]	Mouse/Rat	[21,23, 25–27]
• Inducible depletion allows selective targeting of microglia (and other long–lived myeloid cells) over short–lived BDMC. • Availability of novel microglial–specific Cre lines allow more selective depletion (e.g., Tmem119).						• Short–lived depletion. • Cognitive impairment. • Variable Cre recombination efficiency. • Cre/loxP leakiness. • TAM–associated side effects. • TAM non–specific activation on estrogen receptor cells. • Cre recombinase toxicity.					
Cd11b–HSVTK	<28 days	90–97%	?	Yes	Yes	Yes	Yes	Yes	No	Mouse	[14–20]
• Treatment withdrawal causes microglia replacement by peripheral cells.						• BBB compromise. • Ganciclovir–associated side effects. • Requires BM transfer (i.e., irradiation) or surgical implantation of osmotic pump Cd11b–HSVTK to circumvent myelotoxicity.					
Clodronate liposomes	<5 days	Variable	Yes	Yes	No	No	No	Yes	No	Any	[31–33]
• Can target specific subsets of macrophages (e.g., i.c.v. administration of mannosylated liposomes targets perivascular macrophages).						• Do not cross BBB: surgery required to target microglia. • Short–lived depletion. • Induces cytokine release, astrogliosis, and blood vessel damage.					
***Csf1r*$^{-/-}$/ *Csf1r*$^{fl/f}$**	Lifelong / indefinite	100%	No	No	Yes	KO	Yes	No	Yes	Mouse	[37,44,54]
• Can be used to transplant exogenous myeloid cells into the brain. • Conditional knockout (using loxP sites and Cre lines) allows spatial and temporal control of microglial depletion.						• Shortened lifespan, unless carefully nurtured. • Exhibit skeletal and neurodevelopmental abnormalities. • Reduced tissue resident macrophages. • Discrepancies in observed abnormalities between different genetic backgrounds in mice.					

Advantages						Disadvantages					
Csf1r $^{-/-}$	Lifelong	100%	No	No	Yes	KO	Yes	No	Yes	Rat	[57]
• No microglia. • Survive into adulthood. • No overt brain abnormalities.						• Reduced myelination. • Reduced tissue resident macrophages.					
CSf1 $^{-/-}$ (***Csf1*** $^{op/op}$)	Lifelong	30%	No	No	Yes	KO	Yes	No	Yes	Mouse	[37–44,61]
						• Majority of macrophages are absent. • Olfactory bulb and fertility deficits. • Abnormal brain development.					
IL34 $^{-/-}$	Lifelong	70%	No	No	Yes	KO	No	No	Yes	Mouse	[47]
• Little effect on other myeloid cells (e.g., blood monocytes and tissue macrophages).						• Depleted langerhans cells resulting in reduced contact hypersensitivity. • Reduced lung dendritic cells.					
CSF1R $^{\Delta FIRE/\Delta FIRE}$	Lifelong	100%	No	No	Yes	KO	Yes	No	Yes	Mouse	[56]
• No microglia. • Developmentally normal, healthy, and fertile. • Survive into adulthood.											
TGF-β1 $^{-/-}$	Lifelong	100%	No	No	Yes	KO	Yes	No	Yes	Mouse	[53]
						• Peripheral delivery of TGF–β required for mouse viability. • Motor deficits/mortality reported from 4–6 months of age. • Neurotransmitter/synapse deficits. • Increased infiltration of peripheral monocytes.					
PU.1 $^{-/-}$	Lifelong	100%	No	No	Yes	KO	Yes	No	Yes	Mouse	[51–52]
						• Fail to develop microglia, resulting in shortened lifespan (unless provided with BM transplant from wild–type mice).					

[a]Abbreviations: BBB, blood–brain barrier; BM, bone marrow; BDMC, BM-derived myeloid cells; CSF1R, colony-stimulating factor 1 receptor; i.c.v., intracerebroventricular; KO, knockout; TAM, tamoxifen.
[b]Can be controlled for with peripheral inhibitors.
[c]Unless administered during development.
[d]The extent of depletion and the cells affected depend on the Cre driver line.

Csf1r-deficient mice [44,60]. Although CSF1R expression is restricted to cells of the myeloid lineage in the adult vertebrate brain, expression in non-myeloid cells remains controversial [58]. *Csf1r* is reportedly expressed by some neurons during development in the mouse brain [48], and loss of CSF1 signaling in these cells and CSF1R^{+} osteoclasts is thought to elicit the gross abnormalities observed in murine *Csf1*$^{-/-}$ and *Csf1r*$^{-/-}$ brains, in addition to defects in craniofacial structure [48,61]. However, further research will be necessary to reconcile the discrepancies in the effects of impaired CSF1R signaling on developmental survival and brain structure across species and across genetic mutations, as well as to what extent microglia are involved in this process.

Pharmacology-Based Microglial Depletion: CSF1R Inhibitors Allow Investigations into Adult Microglial Homeostasis

Although the first generation of CSF1R-associated knockout mice (e.g., *Csf1*$^{-/-}$, *Csf1r*$^{-/-}$, *Csf1*^{op}/*Csf1*^{op}) revealed that CSF1R signaling is essential for the development of microglia, studies into the role of this axis in the adult mouse CNS were limited. Our research group initially

identified two CSF1R inhibitors (CSF1Ri), PLX3397 and PLX647, that cross the BBB, and tested their ability to prevent microglial proliferation in mouse models of lipopolysaccharide (LPS)-induced neuroinflammation. However, instead of inhibiting proliferation, we observed robust reductions in microglial numbers throughout the brain relative to vehicle-treated mice, demonstrating microglial dependence on CSF1R signaling in adult mouse CNS tissue [62]. Subsequent formulation of PLX3397 in rodent chow at a dosage of 290 ppm afforded a non-invasive method for CSF1R inhibition, as resultant CNS inhibitor concentrations in the low micromolar range induced 50% depletion of microglia within 3 days of treatment, and up to 99% depletion by 3 weeks in adult WT mice. Increasing the dose of PLX3397 in chow to 600 ppm induced ~99% depletion of microglia within 7 days [63] (Figure 1). Of clinical relevance, PLX3397, or pexidartinib, has been granted US FDA approval as a drug treatment for tenosynovial giant cell tumors, making it an ideal candidate for application to other disorders involving myeloid dysfunction [64]. However, limitations of this compound include its relatively poor CNS penetrance (~5% [62]) and potential off-target effects on related receptor tyrosine kinases c-Kit and Fms-like tyrosine kinase 3 (FLT3) [64]. Additional inhibitor screening led to the identification of PLX5622, which exhibited both a higher specificity for CSF1R and improved brain penetrance (20%) than PLX3397, and delivery in chow at 1200 ppm induced the depletion of >80% of microglia within 3 days in adult mice [65]. Research utilizing these inhibitors has reported microglial loss throughout the CNS, including the murine brain parenchyma, spinal cord, and retina [66–68], owing to microglial cell death [62] rather than to downregulation of microglial markers [69].

Increasingly widespread use of PLX3397 and PLX5622 has shown that this method of microglial depletion can be fast-acting and versatile, and paradgims utilizing these inhibitors have been broadly applied to various mouse models of CNS disease and/or injury [65,66,69–74], as well as to other species including non-human primates [75], and humans [76]. Specifically, microglial densities were quantified in resected glioblastoma tissue from patients treated orally with 1000 mg/day PLX3397 for 7 days, and compared to historical samples from the same patients, revealing moderate but promising effects on microglia [76]. Futhermore, CSF1Ri induced microglial death without subsequent inflammation, cytokine storm, or BBB damage, and had no detectable negative effects on behavior, cognition, or general health when tested on mouse models and non-human primates [62,75]. It should also be noted that the regulation of the extent of CNS CSF1R inhibition through the formulation of different inhibitor concentrations in chow allows titration of microglial depletion (i.e., 20%, 50%, or 99% elimination depending on the dose), thus permitting comprehensive manipulation of the microglial population [62,63,65,77]. Microglial depletion is sustained for as long as treatment is continued – for example, we recently attained 6 months of uninterrupted microglial depletion (~99%) with PLX5622, including in the **5×FAD mouse model** of AD [65] (Table 1).

Together, this work demonstrates that CSF1Ri in mice have given researchers an unprecedented ability to study both (i) the importance of CSF1R signaling in adult microglia, and (ii) the effects of microglial depletion for any duration on CNS development, homeostasis, and disease. Other putative specific CSF1Ri (e.g., JNJ-40347527 and GW2580) are available, and studies have shown that low concentrations of these CSF1Ri in the CNS can alter microglial phenotype (i.e., cell proliferation and self-renewal) without extensive cell loss, in turn offering therapeutic benefits in mouse models of neurological disorders [78–81]. In these studies, administration of low-dose CSF1Ri could attenuate synaptic and neuronal degeneration, reduce neuroinflammation, slow disease progression, and improve cognitive and behavioral function in mouse models of AD [78,81], PD [82], ALS [83], prion disease [80], lupus [84], and spinal cord injury [85]. The lack of observed microglial cell death when using these inhibitors has been attributed to their poor BBB penetrance and thus low exposure to CNS CSF1R, as seen with antibodies that do not

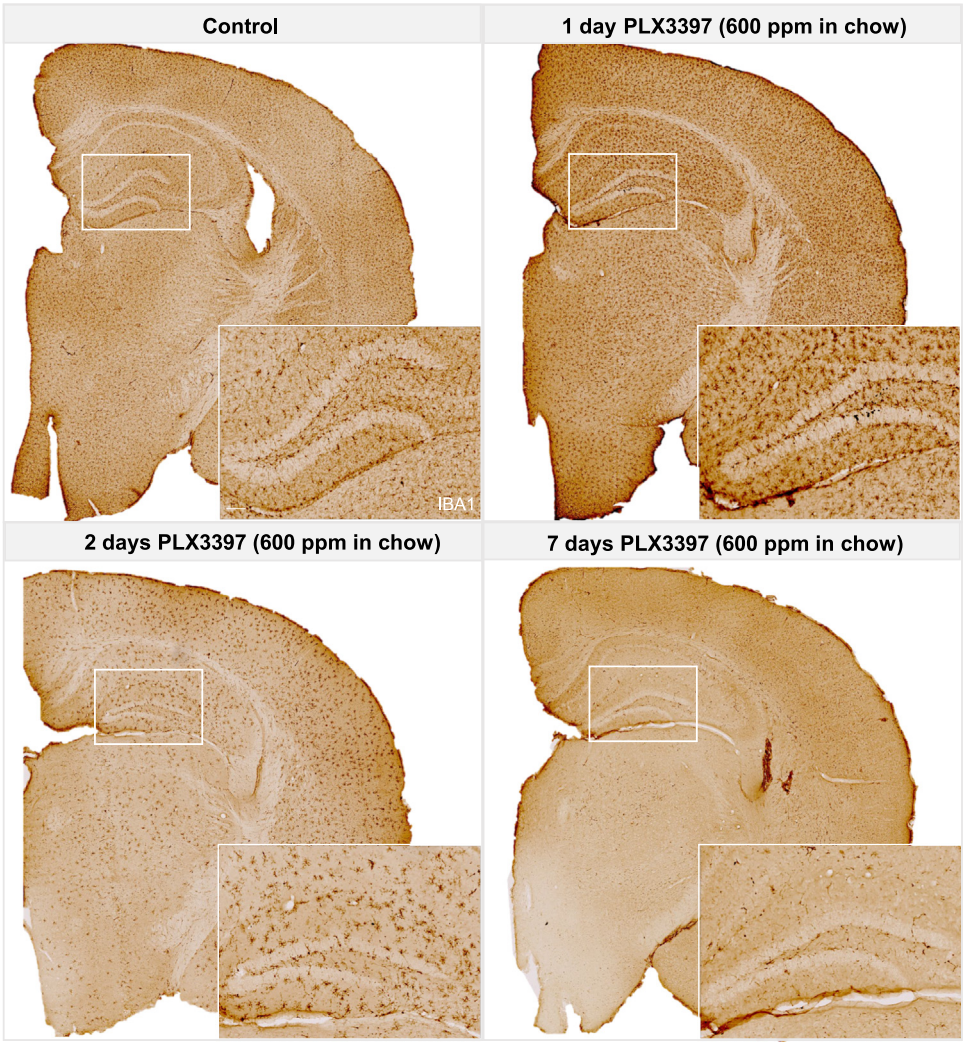

Figure 1. Widespread Depletion of Murine Microglia with Orally Administered Colony-Stimulating Factor 1 Receptor (CSF1R) Inhibitors. For illustrative purposes, wild-type mice aged 2 months were treated with the CSF1R inhibitor PLX3397 (600 ppm in chow) for 1, 2, or 7 days. Controls were treated for 7 days with vehicle chow. To visualize microglial depletion, representative micrographs of brain sections stained via chromogenic immunohistochemistry using the common microglial marker ionized calcium binding adaptor molecule 1 (IBA1) are shown. Higher-resolution images are provided as insets. Scale bar, 120 μm.

readily cross the BBB [86,87]. In line with this, reports in mice show that higher doses or longer exposures (i.e., months vs days of treatment) of these CSF1Ri (i.e., GW2580 and BLZ945) do indeed lead to microglial cell loss instead of reduced proliferation [88,89]. In adult *Cx3cr1*$^{GFP/+}$ mice, 7 days of treatment with BLZ945 (200 mg/kg/day) resulted in a significant reduction of parenchymal microglial cells in areas of white matter [89]. Furthermore, recent studies show that peripheral administration of function-blocking antibodies against the cognate ligands CSF1 or IL-34 induce brain region-specific microglial ablation in a dose-dependent manner [74]. In adult mice, anti-CSF1 antibodies effectively deplete white matter microglia, whereas anti-IL-34 antibodies effectively deplete grey matter microglia, consistent with regional ligand expression [74]. Moving forward, these antibodies could allow even more nuanced investigations of microglial populations in health and disease.

Caveats and Controls for CSF1Ri-Mediated Microglial Depletion

Pharmacological CSF1R inhibition is a highly effective and versatile method to achieve microglial elimination; however, investigators should be aware of the associated caveats (summarized in Table 1). Although this method is surgically noninvasive, treatment is dependent on peripheral administration of a small molecule which, like any exogenously administered agent (e.g., tamoxifen or ganciclovir), may have off-target effects. In addition to the robust dependency of microglia on CSF1R signaling, CSF1R is expressed on all myeloid cells throughout the body, and thus CSF1Ri can also interfere with signaling through this receptor in peripheral myeloid cell populations. In both humans and mice, PLX3397 (at 1000 mg in humans and 400 mg/kg in mice) has been reported to decrease a specific subset of circulating monocytes [76,90]. We and others have not observed significant alterations in blood and spleen myeloid cell populations (including circulating monocytes and tissue macrophages) utilizing either PLX3397 (at a dose of 290 mg/kg or lower) or the more selective inhibitor PLX5622 in mice; however, these studies are limited, and require further examination of circulating and other peripheral tissue-resident macrophage populations [65,71,91–95]. Nonetheless, any effects on peripheral myeloid cell populations can be at least partly controlled for by including treatment groups with classes or dosages of CSF1Ri that attain little to no BBB penetrance in healthy adult mice, such as PLX73086 [66], Ki20227 [96], or PLX3397 at 75 ppm [97]. It should be noted that the use of CSF1Ri in development shares similar limitations to studies utilizing global constitutive *Csf1r*$^{-/-}$ mice, resulting in abnormal phenotypes such as bone malformations and altered hypothalamic-related processes [98]. An important recent discovery showed that intraperitoneal postnatal BM transplant can rescue ~50% of *Csf1r*$^{-/-}$ mouse pups, which again typically do not survive past weaning age, and can partially rescue many *Csf1r*$^{-/-}$-associated phenotypes, including loss of microglia [44,60,99]. In theory, such implementation could have similar effects in developmental CSF1Ri studies, and thus warrants further research as a complementary technique.

What Have We Learned from Microglial Depletion Models?

Although microglia have well-established roles in immunity and immunosurveillance, other homeostatic functions have come to light, including essential roles in synaptic refinement and sculpting, as well as in **neurogenesis** [3,100,101]. Studies in mice indicate that microglia are involved in regulating learning-induced synaptic modifications (in early adulthood) [27] and in phagocytosing apoptotic neuronal progenitors in CNS neurogenic niches [102,103]. Microglial depletion models have been widely utilized to enhance our knowledge of microglial homeostasis, and recent studies using CSF1Ri have shown that microglial depletion in the healthy adult mouse brain does not induce behavioral or cognitive impairments, nor does it exact specific pathologies or overt phenotypes on the CNS [24,62]. Thus, it appears that microglia are in this sense dispensable in the healthy adult brain, outside their pivotal roles in immunity [104]. Microglia do, however, appear to be essential for proper brain development, at which time they adopt a spectrum of unique phenotypic and functional roles [3]. During embryonic and postnatal development, depletion studies in mice have shown that microglia promote the survival of developing neurons through the release of neurotrophic factors [105]; moreover, imaging and genetic studies have demonstrated that microglia remove apoptotic neurons and debris [106,107], and engage in synaptic pruning and neuronal circuit maturation [108–110]. Any disturbance in the ability to carry out these roles can lead to lifelong impairments in cognition and behavior [100,111].

Despite the lack of an overt phenotype produced by chronic microglial elimination, more nuanced effects have been identified that indicate microglial homeostatic function. Several **oligodendrocyte** precursor cell (OPC)-related genes (e.g., *Cspg4*, *Pdgfra*) are downregulated with PLX3397-mediated microglial depletion, accompanied by increases in mature oligodendrocyte-expressed genes (e.g., *Cldn11*, *Cnp*, *Mag*) [63], consistent with previous reports utilizing the inhibitors

PLX3397, PLX5622, and BLZ945 in mice [89,112]. A recent report showed that PLX5622-resistant microglia in *Cnp*$^{-/-}$ mice (a mouse model for white matter inflammation and catatonia) display a highly inflammatory phenotype leading to OPC phagocytosis and reduced OPC cell numbers relative to WT mice [113]. However, studies indicate that these OPC-related effects are dose- and CSF1Ri specificity-dependent, and that long-term oral administration of CSF1Ri has no effect on mature oligodendrocytes or myelin protein expression in adult mice [112]. Although microglial regulation of astrocyte reactivity is apparent [114], neuronal gene expression is largely unaffected by CSF1Ri in the healthy mouse brain [65]. Together with the loss of microglial gene expression with chronic CSF1Ri treatment, this suggests that the absence of microglia from the adult brain does not inherently elicit pathologic or immune mechanisms in other cells [65].

Despite the lack of clear changes at the transcriptional level, however, neuronal alterations have been documented after CSF1Ri treatment, including increases in neuronal dendritic spine densities, synaptic markers, and neurogenesis compared to untreated controls [70,101,115,116]. One of the largest and most consistent effects we observe with microglial elimination via PLX3397 and PLX5622 in mice is a brain-wide increase in **perineuronal nets** (PNNs) [117] – specialized extracellular matrix (ECM) structures that form primarily around **GABAergic interneurons** to effectively 'lock' synapses in place and provide synaptic stability [118]. PNN structural modification regulates interneuron firing rate [119], synaptic transmission [120], and the molecular composition of synapses [121]; thus, homeostatic and disease-related interactions between microglia and the ECM may have implications for related neuronal and synaptic physiology. This is underscored by recent work in mice showing that ECM clearance by microglia in response to neuronal IL-33 can promote synaptic remodeling and plasticity because genetic deletion of neuronal IL-33 (*Il33*$^{flox/flox}$*:Syn1*^{cre}), or inducible myeloid-specific deletion of cognate microglial receptor IL1RL1 (*Il1rl1*$^{flox/flox}$*:Cx3cr1*$^{cre/+}$) resulted in a redistribution of ECM material from microglia to synapses, that in turn was associated with reduced hippocampal dendritic spine densities and impaired remote fear memory recall precision relative to controls [122].

Microglial elimination studies have also provided insight into microglial population dynamics. We have shown that, upon cessation of CSF1Ri treatment (i.e., PLX3397 or PLX5622) in adult WT mice, the microglial compartment has a remarkable capacity to regenerate and repopulate the brain with new microglial cells, and this occurs without contribution from peripheral cells [62]. These repopulating microglia eventually become indistinguishable from their original counterparts in regards to density, morphology, tiling pattern, gene expression, and immune response [123]. Furthermore, CSF1Ri-treated (i.e., PLX3397 and PLX5622) mice can undergo multiple depletion–repopulation cycles in the CNS, given adequate time between treatments [63]. In our original study characterizing microglial elimination–repopulation, two proliferating cell populations were identified: surviving microglia and an unknown non-myeloid cell [62]. Subsequent studies using lineage-tracing approaches via *Cx3cr1*$^{creER/+}$ mice have shown that microglial self-renewal is driven solely by the proliferation of surviving microglia [5]. In addition, although microglial repopulation occurs in virtually every depletion model, it displays differential sources and kinetics based on the mode of depletion. For example, repopulating microglia are found to proliferate in clusters following DT-mediated ablation [23], whereas brain-wide, uniform proliferation is observed after CSF1Ri treatment in mice [62]. Of note, researchers have found that perturbations in BBB integrity or immune activation (e.g., cytokine storm) can allow engraftment of peripheral myeloid cells in place of endogenously repopulating microglia. This occurs either when irradiation is combined with CSF1Ri treatment [54] or toxin depletion in *Cx3cr1*$^{creER/+}$*–iDTR* mice [23], or in the absence of irradiation upon tamoxifen or ganciclovir delivery in *Cx3cr1*$^{creER/+}$*–DTA* mice [26] or *CD11b–HSVTK* mice [19], respectively. Together, these tools provide remarkable control of the brain myeloid population and may enable more comprehensive neuroimmunological inquiries.

Microglial depletion paradigms have afforded much knowledge on the roles these cells play in disease pathogenesis, injury, and recovery. Investigating microglial function via their removal or replacement in animal models of brain disorders has provided insights into the evolving spectrum of their context-dependent phenotypes, which may be beneficial or detrimental [24,124]. The timing of CSF1Ri treatment and microglial depletion relative to disease/injury onset, as well as the type of disease model itself (e.g., acute, neurodegenerative), should be duly considered when interpreting and designing such studies. For example, on the one hand, pharmacological elimination of microglia following brain injury or neurodegenerative pathology development has been shown to be protective [70,77]. In these studies, microglial elimination has improved behavioral-, cognitive-, inflammation-, and synaptic-related outcomes in mouse models of neuronal injury and AD [70,77]. Moreover, CSF1Ri treatment before and/or during symptom/pathology onset is also widely beneficial in several progressive neurodegenerative models [65,81,117,125]. Indeed, recent studies have shown that microglial depletion using PLX3397 and/or PLX5622 fully prevents neuronal loss and atrophy in mouse models of AD [69], **tauopathy** [90], and Huntington's disease [117], suggesting that the microglial response to extracellular plaques or intraneuronal aggregates might primarily drive neurodegeneration. However, on the other hand, increased neuronal damage can accompany microglial elimination when it occurs during (rather than after) the acute stages of injury [70] or before ischemic stroke [71,126], whereas the opposite occurs in hemorrhagic stroke [73] and traumatic brain injury [127]. Whether microglial repopulation/inhibitor cessation occurs before experimental readout also factors into the interpretation of the results; for example, microglial repopulation– but not depletion can improve recovery following neuronal injury [115] and traumatic brain injury [128,129], as well as reverse age-induced long-term potentiation (LTP) and cognitive deficits in aged mice relative to controls [101]. In addition to providing guidance in implementing microglial depletion paradigms, these results once more emphasize the fundamental roles microglia play in health and disease, and suggest clinical contexts in which targeting microglia might have beneficial outcomes.

Recently, a report elucidated the functional role of microglia in mediating the loss of **contextual fear memory** via **complement**-dependent removal and lysosomal degradation of synaptic elements (i.e., **PSD95**) in healthy adult mice [130]. This study parallels our previous work showing enhanced spatial memory in healthy mice following microglial depletion via PLX3397 or PLX5622 [62,65,70]. Furthermore, some of the primary effects of microglial elimination in mice that we have consistently found are increases in PSD95 and **synaptophysin** synaptic staining [70], enhanced dendritic spine densities [70,115], and unimpaired or improved performance in memory-related tasks relative to controls, even after 6 months of chronic microglial depletion [65]. Similarly, microglial depletion consistently induces increased neurogenesis [101] and upregulation of PNNs [117], suggesting multiple mechanisms by which microglia might impact cognition and brain function.

Concluding Remarks

Pharmacological CSF1R inhibitors provide unprecedented control over microglial population dynamics in animal models, attaining rapid and sustained depletion through oral administration in chow, without inducing notable compensatory mechanisms or debilitating health effects. Indeed, perhaps one of the most interesting findings from CSF1Ri research is that microglial loss does not induce any overt phenotypic or cognitive abnormalities in otherwise healthy adult mice. We and others [104] hypothesize that changes in microglial phenotype associated with canonical 'activation' are far more detrimental to parenchymal homeostasis, and thus cognition, than the absence of unstimulated microglia expressing homeostatic phenotypes – or, as we say, 'bad' microglia are worse than no microglia. It is fairly clear that this is not entirely the case in development because early microglial loss results in brain abnormalities [37,40,48,59,98,116] and/or premature

Outstanding Questions

Can we formulate CSF1Ri to selectively target other CNS myeloid cell populations (i.e., meningeal, perivascular, or choroid plexus macrophages)?

What are the phenotypic and therapeutic effects of modulating microglia with CSF1Ri doses that do not result in cell depletion?

Can CNS delivery of CSF1Ri be improved?

What is the relationship between loss of *Csf1r* function (owing to haploinsufficiency or mutation in *Csf1r*) and chronic CSF1R inhibition in the vertebrate brain?

Can we combine microglial elimination with targeted genetic approaches to better understand the mechanisms underlying microglial cell biology versus the effects of their global loss in the brain?

Are the beneficial effects of CSF1R modulation seen in rodent models of disease translatable to humans?

death [53] across numerous preclinical models and species, an observation mirrored by human case reports [59]. In models where discrepancies arise in this regard, such as the *Csf1r*$^{\Delta FIRE/\Delta FIRE}$ mice which reportedly do not present overt neurological disruption despite a lack of microglia from birth [56], we predict that more nuanced measures of synaptic connectivity (e.g., dendritic spine complexity or LTP) will reveal consistent deficits. If not, it is possible that compensatory mechanisms have been activated and require further study [56]. Either outcome would underscore the necessity of the processes carried out by microglia for normal brain development.

Although brain-wide elimination of microglia is clinically unlikely, it is an effective approach to elucidate the roles of these cells in any given biological process. These studies, together with the recently expanding analysis of microglia at single-cell resolution [6,7], have given greater insight into brain myeloid biology than was previously possible in neuroimmunology research. Future preclinical studies combining both microglial depletion and single-cell profiling may elucidate the differential effects of CSF1Ri on specific microglial subtypes (see Outstanding Questions). Furthermore, CSF1Ri at concentrations that do not induce microglial death but modulate the phenotype may – depending on the microglial response and pathological context – confer benefits in disease models, offering more clinically relevant indications (see Outstanding Questions). The ability to replace microglia through cessation of CSF1Ri treatment [123] also has obvious clinical merit, bolstered by restorative effects in aging [101] and injury [115] mouse models, and warrants continued investigation. Because CSF1Ri effectively depletes microglia in humans [76] and nonhuman primates [75], this approach is translationally relevant and, together with the recent FDA approval of PLX3397, it is likely that CSF1Ri will remain a mainstay of basic and biomedical microglial research for some time to come.

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