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REVIEW

Phenotypic impacts of CSF1R deficiencies in humans and model organisms

David A. Hume¹  | Melanie Caruso¹ | Michelle Ferrari-Cestari¹ | Kim M. Summers¹ | Clare Pridans² | Katharine M. Irvine¹

¹Mater Research Institute, University of Queensland, Woolloongabba, Queensland, Australia

²Centre for Inflammation Research, The University of Edinburgh, Edinburgh, United Kingdom

Correspondence

Professor David A. Hume, Mater Research Institute-University of Queensland, Translational Research Institute, 37 Kent Street, Woolloongabba, Qld 4102, Australia.
Email: David.Hume@uq.edu.au

Abstract

*M*φ proliferation, differentiation, and survival are controlled by signals from the *M*φ CSF receptor (CSF1R). Mono-allelic gain-of-function mutations in *CSF1R* in humans are associated with an autosomal-dominant leukodystrophy and bi-allelic loss-of-function mutations with recessive skeletal dysplasia, brain disorders, and developmental anomalies. Most of the phenotypes observed in these human disease states are also observed in mice and rats with loss-of-function mutations in *Csf1r* or in *Csf1* encoding one of its two ligands. Studies in rodent models also highlight the importance of genetic background and likely epistatic interactions between *Csf1r* and other loci. The impacts of *Csf1r* mutations on the brain are usually attributed solely to direct impacts on microglial number and function. However, analysis of hypomorphic *Csf1r* mutants in mice and several other lines of evidence suggest that primary hydrocephalus and loss of the physiological functions of *M*φs in the periphery contribute to the development of brain pathology. In this review, we outline the evidence that CSF1R is expressed exclusively in mononuclear phagocytes and explore the mechanisms linking *CSF1R* mutations to pleiotropic impacts on postnatal growth and development.

KEYWORDS

CSF1R, *M*φs, neurodegeneration, osteoclasts, transgenic

1 | INTRODUCTION

*M*φs are an abundant cell population in all major organs and adapt at each location to perform specific functions in physiology and homeostasis.^{1–4} *M*φ proliferation and differentiation is controlled by signals from the *M*φ CSF receptor (CSF1R), in response to 2 alternative ligands, *M*φ CSF1 and IL-34. *M*φs generated in vitro from monocytes or bone marrow progenitors, by cultivation in CSF1, have been widely used as models for the study of *M*φ biology in multiple species.^{5–8} In the mouse, *M*φs also depend upon exogenous CSF1 for survival.⁹ In other species, including humans, rats, pigs, sheep, goats, cattle, horses, water buffalo, and even chickens, CSF1 is induced during *M*φ differentiation. Mature *M*φs themselves express high levels of CSF1 mRNA and are effectively autocrine for the pro-survival signal (data in ref. 10–13). Notwithstanding the apparent differences in expression

among species, the 2 functional ligands, CSF1 and IL-34 are conserved across species from chicken and fish through to humans^{14–17} and an intronic enhancer that regulates *CSF1R* expression is conserved from reptiles to humans.¹⁸ The *CSF1R* gene was originally recognized by its relationship to the transforming oncogene of a feline leukemia virus¹⁹ and was known as the *Fms* protooncogene. Aside from the functions in *M*φs, in both mouse and human (and in other mammals; www.biogps.org) *CSF1R* is also expressed in placental trophoblasts driven by a separate promoter and transcription start site to those used in *M*φ lineage cells.²⁰

In 2011, the human *CSF1R* gene was recognized as the site of point mutations underlying autosomal dominant adult-onset leukoencephalopathy with axonal spheroids and pigmented glia (ALSP)²¹ (MIM: 221820, also known as HDLS). Subsequent studies have revealed many additional *CSF1R* mutations associated with ALSP

Abbreviations: ALSP, adult-onset leukoencephalopathy with axonal spheroids and pigmented glia; BMDM, bone marrow-derived *M*φ; GH, growth hormone.

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(reviewed in ref. 22 and 23) and unified the diagnosis with a number of other disease entities with similar presentation (e.g., pigmented orthochromatic leukodystrophy or POLD). The brain pathology and symptoms of ALSP vary significantly between affected individuals and the disease has been misdiagnosed antemortem as various other dementias and neurodegenerative diseases. For example, Sassi et al.²⁴ identified 3 likely pathogenic CSF1R mutations in a cohort of 465 late-onset Alzheimer's patients.

A distinct recessive disease has more recently been associated with loss-of-function alleles at the *CSF1R* locus.^{25,26} Patients lacking *CSF1R* in the recessive disease had almost complete loss of microglia, the *Mφ*s of the brain, as well as defects in skeletal development and osteosclerosis. As far as we are aware, no patients have been described in whom there is homozygous loss-of-function mutation in either *CSF1* or *IL34*. The contact amino acids involved in interactions in the *CSF1*-*CSF1R* and *IL-34*-*CSF1R* complexes have been dissected in crystal structures.^{27,28} In the large human exome sequence collection ExAC (exac.broadinstitute.org), there are no non-synonymous mutations in *CSF1* that potentially affect binding of *CSF1* to the receptor, and only 2 rare variants in *IL-34* (E111K and W116G) that could alter *IL34* binding. However, there is likely to be expression variation between individuals. SNPs within the *CSF1* locus have been associated with Paget's disease, an abnormality of bone resorption,²⁹ most likely associated with overexpression of *CSF1* and excessive osteoclast activation. The level of circulating *CSF1* in a very large cohort of coronary artery disease patients was correlated with distinct and relatively common *cis*-acting variants at the *CSF1* locus and in turn with susceptibility to disease.³⁰ In this article, we will critically review the interpretation of studies of *Csf1*, *Il34*, and *Csf1r* mutations in experimental animals and their relevance to the human genetic diseases.

1.1 | The cell-type and tissue specificity of *Csf1r* expression

A key piece of knowledge required to interpret the impact of *Csf1r* mutations is the site of transcript and protein expression. The main site of expression of *Csf1r* (aside from expression in placental trophoblasts) is undoubtedly in cells of the monocyte-*Mφ* lineage. Reporter transgenes driven by the *Csf1r* promoter have been used to locate *Mφ*s throughout embryonic development and in tissues of adult mice,³¹⁻³³ rats,³⁴ sheep,³⁵ and even chickens.^{14,15} The same mouse *Csf1r* promoter driving Cre recombinase has also been used in lineage trace experiments in the embryo³⁶ without any evidence of expression outside myeloid lineages.

During mouse embryonic development, *Csf1r* mRNA is first detected in the ectoplacental cone early after implantation and in isolated *Mφ*-like cells in the yolk sac.³⁷ Localization of *Csf1r* mRNA by whole mount in situ hybridization is consistent with restriction to myeloid cells in the embryo.³⁷ Although they are abundant and actively involved in clearance of apoptotic cells,³⁷ *Mφ*s in the embryo are not required for organogenesis. Ablation of *Csf1r*-dependent *Mφ*s by anti-*CSF1R* treatment of the mother has no effect on embryonic

development³⁸ and *Csf1r*-deficient mice and rats are indistinguishable from their littermates at birth.^{39,40}

The restriction of *Csf1r* expression to cells of the *Mφ* lineage is also supported by network analysis in both mouse and human systems. In mouse development, the appearance of *Csf1r* mRNA is strongly correlated with expression of other known *Mφ* markers in a time course of embryo gene expression.⁴¹ In the large promoter-based transcriptomic atlas produced by the FANTOM Consortium, there is a single cluster of *Mφ*-specific transcription start sites in both mouse and human.¹³ There is no detectable *CSF1R* expression in non-myeloid primary cells or cell lines of multiple lineages. Interestingly, *CSF1R* mRNA is also tightly correlated with a *Mφ* signature in gene expression profiles from a wide range of human solid tumors⁴² suggesting that it is exclusively expressed by tumor-associated *Mφ*s and ectopic expression in tumor cells is not common. The molecular basis for *Mφ*-specific transcription of *Csf1r* and reporter gene expression has been reviewed elsewhere.^{1,20} An inducible Fas-based suicide gene driven by the *Csf1r* promoter (the so-called MAFIA mouse) has been applied to functional studies of *Mφ*s in vivo without evidence of ectopic expression or adverse impacts on other cell types.⁴³

In spite of this compelling evidence that *Csf1r* expression is restricted to cells of the monocyte/*Mφ* lineage, the recent studies of the *CSF1R* homozygous mutation in human patients^{25,26} and reviews of *CSF1R* roles in embryonic and postnatal development (e.g., ref. 44) cite a small number of studies that claim to demonstrate expression of *Csf1r* in non-hematopoietic cells including neuronal progenitors, intestinal and renal epithelial cells, and cells of the female reproductive system. If these reports are correct, then some of the pleiotropic impacts of *CSF1R* mutations in patients and experimental animals might be attributed to defects in non-myeloid cells. It is therefore timely to re-evaluate the evidence from these reports for non-myeloid expression of *CSF1R*.

There are several caveats to each of the studies claiming functional expression of *CSF1R* outside the mononuclear phagocyte lineage, notably in relation to the specificity of anti-*CSF1R* Abs. In the mouse brain, Sierra et al.⁴⁵ provided detailed evidence that the *Csf1r*-EGFP reporter gene produced by our laboratory³² is restricted in its expression to microglia and perivascular *Mφ*s at all stages of postnatal development and in injury and ageing models. Using additional *Csf1r* reporter genes in mice and distinct anti-*CSF1R* Abs, we³¹⁻³³ and others^{46,47} have shown that expression of the transgene and *CSF1R* protein is restricted to microglia and *Mφ*s at all stages of brain development. By contrast, Nandi et al.⁴⁸ reported that *Csf1r* mRNA was expressed in neuronal progenitors and used Ab staining to demonstrate high levels of expression of *CSF1R* protein in these cells in the early postnatal period. The specificity of their Ab binding was based upon lack of binding to *Csf1r*^{-/-} mouse brains, but curiously, the Ab did not appear to detect microglia in wild-type mice. Their study would suggest quite high levels of *Csf1r* mRNA in the developing mouse brain. This is clearly not evident from analysis of deep RNAseq data, including the time course of embryonic development⁴¹ in which expression is low and tightly correlated with increased expression of other known *Mφ*-specific transcripts. In detailed network analysis of the

transcriptomes of isolated cells from mouse and human brain, *Csf1r* is clearly part of a microglia-M ϕ signature.^{49,50}

Based upon conditional deletion of *Csf1r* with a *Nestin*-cre transgene and direct impacts of added CSF1 or IL-34 to "microglia-free" forebrain cultures (selected based upon *Nestin*-EGFP expression), Nandi et al.⁴⁸ proposed that CSF1 acts directly on neuronal stem cells. The interpretation of these studies depends on the view that the *Nestin* promoter is not active in microglia. However, *Nestin* mRNA is detectable in isolated microglia at levels higher than total brain (see data in ref. 13 and www.biogps.org). A subsequent study⁵¹ also used *Nestin*-cre to provide evidence that following depletion with a CSF1R kinase inhibitor (see below) microglia repopulate from a *Nestin*-expressing progenitor. The transient expression of *Nestin* in microglia self-renewing following ablation was confirmed by another group using a different model.⁵²

The most widely cited study claiming neuronal expression of *Csf1r*⁵³ investigated the potential of CSF1R ligands as treatment for dementia and claimed that systemic administration of CSF1 or IL-34 could ameliorate neurotoxicity associated with excitotoxic injury. The authors were not able to detect specific staining for CSF1R protein in wild-type brain that was absent in *Csf1r*^{-/-} mice using any of 6 commercially available polyclonal anti-mouse CSF1R Abs. However, based upon in situ hybridization, they reported expression of *Csf1r* mRNA in scattered neurons in normal uninjured mouse brain, notably in the neuron-dense hippocampal pyramidal layers, and an apparent increase in labeled cells in this region following kainic acid-induced injury. The underlying assumption was that there are no microglia among the neuronal cells. Using an amplified *Csf1r*-EGFP reporter gene, we detect positive cells in the same regions, but they clearly resemble microglia (Fig. 1A). Both this transgene and our *Csf1r*-EGFP and *Csf1r*-mApple transgenes are stringently restricted to CD45⁺ hematopoietic cells in digests of brain, and within that set, co-expressed with the myeloid marker CD11b.³¹⁻³³ The study by Luo et al.⁵³ used 2 other *Csf1r* reporter gene systems; the MAFIA mouse (which has an EGFP reporter separated by an internal ribosomal re-entry site⁴³) and a conditional *Csf1r*-Cre-dependent EGFP reporter, to demonstrate apparent induction of *Csf1r* in injured neurons. They detected EGFP by Ab staining rather than direct imaging of EGFP fluorescence, so even if the signal reflects some level of inducible promoter activity, the signal is not quantitative. Finally, Luo et al.⁵³ generated a conditional knockout by crossing a floxed allele of *Csf1r* to *CaMKIIA*-cre to delete the *Csf1r* gene in neurons. These mice were reported to be more susceptible to kainic acid injury. But the controls in this case were mice that lacked the *CaMKIIA*-cre transgene. There is a need for caution in interpreting both this result and those obtained with *Nestin*-cre. High-level expression of cre recombinase can clearly have impacts on cellular function.⁵⁴ It is entirely possible that expression of cre in neurons directly impacts their functions including the production of CSF1 and IL-34 and the sensitivity to toxic challenge. In overview, we consider there is compelling evidence for the exclusive expression of *Csf1r* mRNA and CSF1R protein in the brain in microglia and M ϕ s.

In the intestine, in *Csf1*^{op/op} and *Csf1r*^{-/-} mice, Paneth cells were lost and there was disordered differentiation of epithelia including

an excess of goblet cells.⁵⁵⁻⁵⁷ In the mouse, *Csf1r* was apparently expressed functionally by Paneth cells and by other epithelial cells in both small and large intestine based upon staining with a commercial rabbit polyclonal Ab against CSF1R.⁵⁵⁻⁵⁷ Conditional deletion of *Csf1r* with a tamoxifen-inducible *Villin*-cre reproduced intestinal epithelial disruptions associated with *Csf1r* mutation leading to the conclusion that *Csf1r* function is intrinsic to epithelial cells.⁵⁵ Consistent with the cautionary note above about cre recombinase, Bohin et al.⁵⁸ subsequently showed that tamoxifen-inducible *Villin*-cre activation per se led to DNA damage and cleavage of cryptic LoxP sites in intestinal stem cells. A secondary concern with the inducible cre recombinase system is that tamoxifen is not a neutral agonist, especially when applied to M ϕ biology (reviewed in ref. 1). The role of *Csf1r* in intestinal homeostasis was reinvestigated in our group by exploring the impact of treatment of mice with a purified blocking rat mAb against mouse CSF1R. Prolonged treatment completely depleted lamina propria M ϕ s and also disrupted epithelial differentiation leading to increased goblet cells and the loss of microfold (M) cells.⁵⁹ However, we did not reproduce the expression of *Csf1r* in epithelial cells. Instead, we demonstrated that the expression of both *Csf1r* mRNA and *Csf1r*-EGFP expression was completely restricted to lamina propria M ϕ s. Figure 1B shows the intimate association between crypt-associated M ϕ s and intestinal stem cells. This conclusion was supported by mRNA analysis of isolated intestinal cell populations. Furthermore, conditional deletion of *Csf1r* using a constitutive *Villin*-cre had no effect on epithelial differentiation. Importantly, Paneth cells were not actually depleted by anti-CSF1R; their expression of markers such as lysozyme and defensins was lost indicating that M ϕ s control their differentiation rather than their survival.⁵⁹ In the rat, we also saw no effect of the *Csf1r* null mutation on the presence of Paneth cells or on overall villus architecture.⁴⁰ We have not yet investigated whether epithelial function is regulated by interactions with M ϕ s in this species.

Resident M ϕ s are an abundant interstitial population in the kidney and Menke et al.⁶⁰ claimed that CSF1 signals directly to renal tubular epithelial cells to promote repair of hypoxic injury. Inducible expression of *Csf1* in damaged tubular epithelial cells has been reproduced by multiple authors. Exogenous CSF1 administration promoted epithelial repair and anti-CSF1R treatment, CSF1R kinase inhibition or genetic deletion of *Csf1* increased the pathology or delayed resolution in several different injury models.⁶⁰⁻⁶³ Menke et al.⁶⁰ claimed that CSF1R protein and *Csf1r* mRNA were detectable in isolated mouse renal epithelial cells and Zhang et al.⁶³ appeared to detect phosphorylated CSF1R in damaged renal epithelium using anti-CSF1R Ab. In the former study, protein detection was based upon the same rabbit anti-mouse CSF1R preparation used by Nandi et al.⁴⁸ The authors noted that *Csf1r* mRNA expression was considerably lower than in M ϕ s and contamination by M ϕ s was not excluded. Based upon conditional deletion using an *Ilgam*-diphtheria toxin receptor transgene, Menke et al.⁶⁰ argued that M ϕ s make a minor contribution to CSF1-dependent repair. Our own study in a similar renal ischemia model, which reproduced the beneficial effect of CSF1 treatment, strongly favors the M ϕ as the mediator of tissue repair and the exclusive responder to CSF1 administration.⁶¹ This conclusion is supported by subsequent

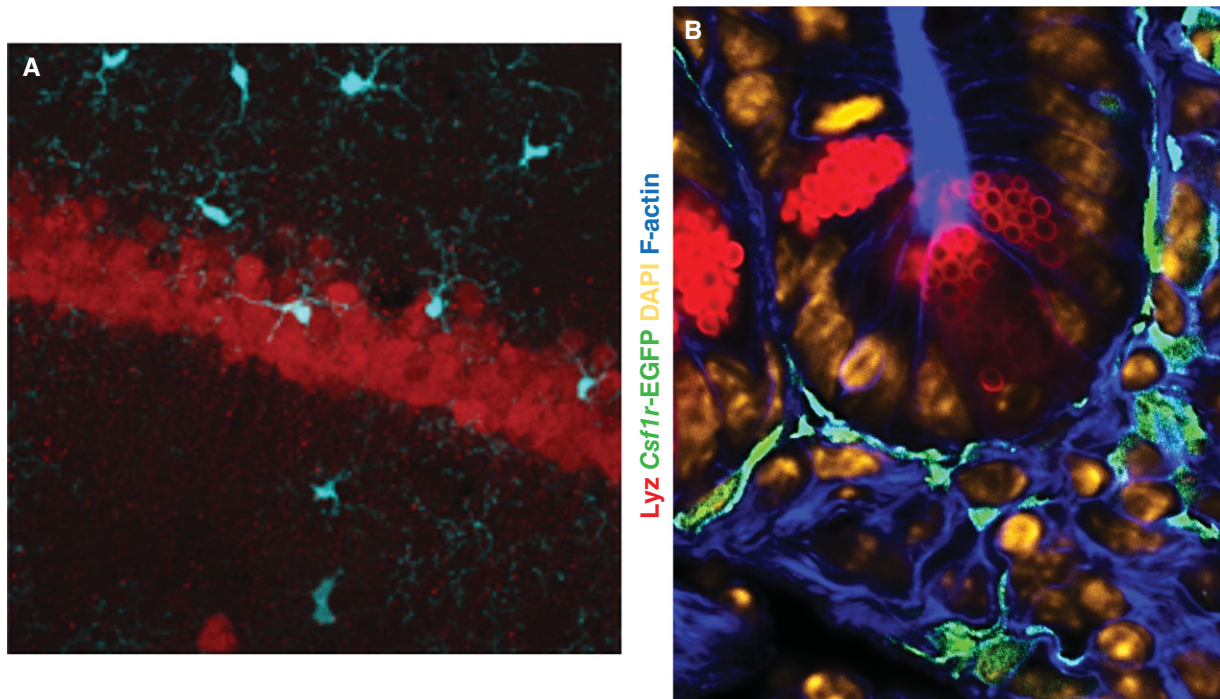


FIGURE 1 Expression of *Csf1r* reporter genes is restricted to macrophages. Panel (A) shows expression of cyan fluorescent protein in microglia in the hippocampus of the brain of MacBlue (*Csf1r*-GAL4VP16/UAS-ECP) mice. The neuronal cells of the pyramidal layer are stained for the neuronal marker NeuN. Reproduced from ref. 33 with permission. In the same study, both ECFP and *Csf1r*-EGFP were detected in disaggregated total brain by FACS exclusively in cells that co-expressed CD11b. Panel (B) shows an optical section of a 3D whole mount of the crypt of a small intestinal villus of *Csf1r*-EGFP mice, stained for the Paneth cell marker, lysozyme. Note the intimate association between EGFP+ cells and the basal membranes of epithelial cells. Phalloidin staining of F-actin is used as a counterstain and highlights the basolateral and apical regions of epithelial cells and the non-M ϕ cells (including endothelial cells and fibroblasts) in the lamina propria. Reproduced and modified from ref. 59 with permission and thanks to Dr. A Sehgal

studies.^{62,63} Menke et al.⁶⁰ described an apparent increase in *Csf1r*-EGFP expression in epithelial cells in response to renal injury but in our view, the apparent increase in EGFP fluorescence was attributable to M ϕ infiltration of the damaged epithelial layers and autofluorescence of tubular casts.⁶¹ In neither study was there any evidence of expression of the *Csf1r*-EGFP reporter genes in undamaged renal epithelium consistent with the original description of the transgene.^{31,32} As in the brain, the increasing abundance of *Csf1r* mRNA in the kidney during embryonic and postnatal development correlated closely with other M ϕ markers.⁴¹ In summary, the claim that CSF1 signals directly to renal tubular epithelial cells in any circumstance is not strongly supported.

There are high-affinity mAbs against murine CSF1R that detect expression in isolated monocytes and progenitors (e.g., ref. 60 and 64) but they have not been used successfully to detect the protein in tissues. In fact, it is intrinsically unlikely that the CSF1R protein is detectable at high levels in tissues because it turns over constantly upon ligand binding (see below). As an alternative, we developed an AF647-conjugated version of CSF1. This protein bound specifically to monocytes isolated from the blood of mice and rats and when injected into mice localized specifically to tissue M ϕ s.^{31,40,65}

The restriction of CSF1R expression to M ϕ s and microglia is also supported by studies using *Csf1r* kinase inhibitors. Elmore et al.⁵¹ reported the almost complete elimination of microglia from the mouse

brain using selective CSF1R inhibitors. There was no evidence of a phenotypic impact and gene expression profiling revealed only the loss of known microglia-associated transcripts. Since this original report, the inhibitor PLX3397 has been used extensively in studies of the functions of microglia in brain and retinal development and homeostasis (e.g. ref. 66–69, and references therein) without any evidence of effects on non-myeloid cells. The lack of effect is actually surprising. Contrary to the way it is portrayed explicitly in many publications, PLX3397 is not a specific CSF1R kinase inhibitor; it is also an effective inhibitor of related kinases KIT and FLT3^{70–72} and likely mediates its effects on microglia in part by interacting with other kinase targets. Another orally available CSF1R kinase inhibitor, GW2580, also penetrates the brain but unlike PLX3397, it prevents microglial proliferation/self-renewal without impacting on survival.⁴⁶

Taking all of these data together, we believe there is no reason to consider *Csf1r* expression outside of myeloid lineages in the interpretation of mutant phenotypes in experimental animals or humans.

1.2 | CSF1R signal transduction

The binding of CSF1 or IL34 to CSF1R and the downstream signaling events have been reviewed in detail by Stanley and Chitu.⁷³ CSF1 signal transduction has mostly been studied in mature M ϕ s, osteoclasts, or cell lines, and mainly in mouse systems where CSF1 is not

produced by *Mφs* themselves and endogenous/autocrine CSF1 signaling is therefore not an issue. For obvious reasons, the CSF1 response is also commonly studied in cells that have been deprived of growth factor to allow up-regulation of surface receptor and the analysis of a synchronous response to receptor ligation.

In broad outline, studies of CSF1 signaling have shown that ligand binding induces dimerization of the receptor and release of the kinase domain from an auto-inhibited conformation leading to initial tyrosine phosphorylation and ubiquitination of a membrane proximal domain. Trans-phosphorylation of individual tyrosine residues in the intracellular domain then provides a scaffold for recruitment of several different effector pathways linked separately to survival, increased cell motility, proliferation, and specific gene regulation. One of these pathways is the classical SOS-GRB2-RAS-RAF-MAPK pathway that was first dissected in detail in the analysis of “sevenless/pointed” pathway in *Drosophila*.⁷⁴ The same pathway from CSF1R through SOS/GRB2, RAS, RAF, and the MAP kinases ERK1/ ERK2 leads in *Mφs* to phosphorylation of the transcription factor ETS2 on the pointed domain. ETS2 then interacts with AP1 transcription factors on a conserved Ras response element to activate transcription of urokinase plasminogen activator (*Plau*)^{75–78} in the same way that AP1 (Jun) interacts with pointed to induce photoreceptors in the *Drosophila* eye.⁷⁹

Following initial signal generation, the SOS-GRB2 complex dissociates from the receptor, there is further tyrosine and serine phosphorylation and a cascade of ubiquitination culminating in degradation of both ligand and receptor in lysosomes.⁷³ Internalization and degradation of the receptor is not blocked by inhibitors of receptor kinase activity.⁸⁰ Further signaling, therefore, requires continuous synthesis of new receptors on the cell surface. The nature of the response to CSF1 depends upon the duration and magnitude of that continued stimulation. Removal of CSF1 from bone marrow-derived *Mφs* (BMDM) at any time leads to rapid loss of expression of *Plau* mRNA⁸¹ and CSF1 must be present continuously in order for cells to enter S phase and subsequently undergo cell division. Because CSF1 is internalized and degraded, binding at 37°C is irreversible and ligand is depleted from the medium. The degradation of CSF1 by proliferating mouse bone marrow-derived *Mφs* is saturated at concentrations that are required to drive entry into the S phase if the cell cycle.⁹ As a consequence of the rapid degradation of the ligand, the dose-response curve for CSF1 action on any measured outcome in cell culture is very steep.⁸² It is actually not a concentration dependence, but a titration of the amount of CSF1 available per cell per hour. If the cells exhaust the supply of growth factor at any time, the signaling cascade terminates. For example, the CSF1-dependent phosphorylation of the MAP kinases ERK1/ERK2 and of their target ETS2 in *Mφs* is sustained for as long as CSF1 is present.⁷⁵ The outcome of signaling also depends upon the cell population. Mature peritoneal *Mφs* are more effective at internalization and degradation of CSF1 than BMDM but do not undergo proliferation; they can compete in vitro for the available CSF1.^{82,83} The *Mφs* of the liver clear CSF1 from the blood⁸⁴ thereby maintaining a low circulating concentration (~20 ng/ml³⁹) that is less than saturating for *Mφ*-mediated clearance by the receptor (~70–100 ng/ml).

The circulating CSF1 concentration in vivo is also sub-stimulatory for monocyte production by the bone marrow and for proliferation and regulated gene expression in resident tissue *Mφs*. As a consequence, the entire mononuclear phagocyte system can respond to increased CSF1 availability. Administration of CSF1⁸⁵ or a CSF1-Fc fusion protein (which has a longer circulating half-life^{7,31,86,87}) leads to both expansion of the blood monocyte pool and proliferation of resident *Mφs* in all organs. It also induces expression by *Mφs* of *Plau* and other target genes with similar regulatory elements (e.g., *Mmp9*). A striking and unexpected consequence of CSF1-Fc treatment is extensive hepatocyte proliferation leading to a rapid expansion of the size of the liver in mice, rats, and pigs.^{34,86,87} This finding indicates that *Mφs* contribute to the homeostatic regulation of liver size relative to body size.⁸⁶ Resident *Mφs* have relatively low rates of proliferation/self-renewal as noted in the original descriptions of the mononuclear phagocyte system (reviewed in refs. 1 and 2). Nevertheless, liver *Mφs*, and most other tissue *Mφ* populations do require CSF1R signals for continued survival and in mice, they are rapidly and sustainably depleted in response to anti-CSF1R treatment.^{64,88}

Like all signaling pathways, CSF1R signaling at multiple levels is subject to feedback controls, many of which are themselves CSF1-inducible (reviewed in ref. 73). They include protein tyrosine phosphatases, serine phosphatases, dual-specificity phosphatases (DUSP), inositol phosphatases, and suppressors of cytokine signaling. Deficiencies in any of these feedback mechanisms can lead to uncontrolled CSF1R signaling and each of them is therefore a potential epistatic modifier of the effect of CSF1R mutations. For example, mutation of the SHP1 hematopoietic tyrosine phosphatase (encoded by *Ptpn6*) in the motheaten mouse leads to constitutive activation of ERK1/2-dependent ETS2 phosphorylation and expression of CSF1R target genes such as *Plau* in *Mφs* and factor-independent survival.⁸⁹ As a consequence of the separation of internalization and degradation from kinase activity and the presence of so many feedback pathways the efficiency of signaling can be controlled at multiple levels. For example, an old study showed that glucocorticoids actually shift the CSF1 dose-response curve for induction of *Plau* and proliferation in mouse BMDM so that cells consume and degrade more CSF1 to produce the same outcome.⁸³ One of the conserved actions of glucocorticoids in mouse and human *Mφs* is to induce the expression of the MAPK kinase inhibitor DUSP1,⁹⁰ which would act to reduce the effective activation of ERK1/2 and efficiency of downstream transmission of the CSF1R signal. The CSF1R/glucocorticoid axis may also be relevant to understanding variable penetrance of CSF1R mutant phenotypes.

1.3 | The phenotypes associated with CSF1, IL34, and CSF1R mutations in mice

Most of our current knowledge of mononuclear phagocyte adaptation is derived from studies of inbred mice. In terms of *Mφ*-specific and inducible gene expression, mouse and human *Mφs* are substantively different from each other.^{8,90} Recent comparative analysis of mouse and human microglial expression signatures also revealed significant differences in gene expression driven by species-specific variation

TABLE 1 Summary of mutations of *Csf1r*, *Csf1*, and *Il34* in rodents

Gene/species	Genetic background	Survival	References
<i>Csf1</i> ^{op/op} mouse	Mixed B6/C3H	Adult, bone phenotype resolves with age	39,92-95
<i>Csf1</i> ^{op/op} mouse	Inbred C57BL/6	Lethal	106
<i>Csf1</i> ^{fl/fl} x Cre	Inbred C57BL/6	Osteopetrosis	157
<i>Csf1</i> ^{tl/tl} rat	Inbred Fischer	Adult, no resolution with age	119
<i>Csf1r</i> ^{-/-} mouse	Mixed 129/Sv and B6/C3H	>80 days, bone phenotype resolves with age	39
<i>Csf1r</i> ^{-/-} mouse	Inbred C57BL/6	Few survive to wean	106
<i>Csf1r</i> ^{-/-} mouse	Mixed FVB/NJ	<4 weeks	47
<i>Csf1</i> ^{dex5/dex5} mouse	Inbred C57BL/6	Few survive to wean	47,102
<i>Csf1r</i> ^{-/-} rat	Mixed DA/SD	>12 weeks	40
<i>Csf1r</i> ^{-/-} rat	Inbred DA	~12 weeks	Unpublished
<i>Csf1r</i> ^{-/-} rat	Outbred SD	>12 weeks	Unpublished
<i>Il34</i> ^{LacZ/LacZ} mouse	Inbred C57BL/6	Adult, fertile	110

in *cis*-regulatory elements and transcriptional regulators.⁹¹ Nevertheless, the biology of CSF1R is conserved in mammals and the mutations in rodents have provided clear insights into M ϕ functions in development. Table 1 summarizes the mutations that have been studied in laboratory animals and the impacts of genetic background.

The osteopetrotic mouse is a spontaneous mutation first described in detail by Marks and Lane.⁹² The original characterization included a failure of tooth eruption, excessive accumulations of bone and compromised marrow cavities, increases in bone matrix formation, and hypophosphatemia associated with primary deficiency of bone-resorbing osteoclasts. Subsequently, 2 groups identified an inactivating mutation of *Csf1* (a single base insertion before codon 88, which results in a premature stop codon after 21 bases) in these mice, now referred to as *Csf1*^{op/op}.^{93,94} The original description of the *Csf1*^{op/op} mouse noted that the osteoclast deficiency and reduction in bone marrow cellularity resolves with age and extramedullary hematopoiesis in the spleen is corrected.⁹⁵ In the spleen of mutant mice, there is a substantial expansion of committed M ϕ progenitors,⁹⁵ but CSF2 (GM-CSF), which can also act upon these shared progenitors, was not required for age-dependent correction.⁹⁶ Age-dependent resolution of the *Csf1*^{op/op} bone phenotype has been attributed to signals from other tyrosine kinase receptors/ligands notably VEGFA and FLT3L.^{97,98} *Csf1*^{op/op} mutation in mice also leads to reduced M ϕ numbers in most organs⁹⁹ and there are numerous pleiotropic impacts including compromised postnatal growth, extensive skeletal abnormalities, defects in sensory neuronal systems, abnormal intestinal organization, Paneth cell deficiency, relative male and female infertility, delayed beta cell development in the pancreatic islets, reduced mammary gland development, defective angiogenesis and lymphangiogenesis, partial B cell deficiency, and altered neurogenesis and brain development (reviewed in refs. 44 and 100). Among many applications, *Csf1*^{op/op} mice have been used to infer the role of CSF1-dependent M ϕ s in the control of cholesterol metabolism and the development of atherosclerosis (reviewed in ref. 101).

Targeted mutation of the *Csf1r* locus in mice by insertion of a reporter gene within exon 3³⁹ revealed a very similar phenotype to the *Csf1*^{op/op} including an expansion of committed M ϕ progenitors

in the spleen. As in the case of the ligand mutation, the bone marrow cellularity and osteoclast deficiency resolved with age. In this original study, the *Csf1r* mutation was generated on the inbred 129SvJ genetic background and crossed to the *Csf1*^{op/op} line on a mixed C57BL6/C3Heb/FeJ genetic background on which the mutation is maintained by the Jackson Laboratories to enable comparison of single and double mutations. With this complex cross, the majority of single or double homozygous *Csf1r*^{-/-} and *Csf1*^{op/op} mice survived to adulthood (at least 80 days). The phenotypic impacts of the 2 mutations were not additive because the double homozygote phenotype was largely indistinguishable from either single homozygote. Both receptor and ligand mutant animals were reportedly able to generate progeny, albeit their fertility was greatly compromised. Small differences in bone phenotype and marginally greater pre-weaning mortality in *Csf1r*^{-/-} mice provided some indication of the existence of a second *Csf1r* ligand. Unfortunately, the original study of the mutation on the mixed genetic background did not examine the brain. Based upon subsequent studies, the *Csf1r*^{-/-} mice that survived beyond weaning were presumably microglia-deficient but not severely affected.

Most subsequent studies of *Csf1r* mutant mice, including a second conditional mutation (deletion of exon 5) made by another laboratory¹⁰² have been carried out on 1 of 2 inbred genetic backgrounds (FVB, C57BL/6) where the perinatal lethality is much more penetrant and very few mice survive to weaning.⁴⁴ In a subsequent study, Dai et al.¹⁰³ examined the bone phenotype of juvenile *Csf1r*^{-/-} mice on an inbred background (FVB/NJ) in greater detail. These mice died within the first 4 weeks of life. In juvenile mutants, they observed chondrodysplasia and defects in collagen matrix organization and mineralization and defects in osteoblasts. Using a novel approach, they transplanted fetal femoral anlagen from *Csf1r*^{-/-} mice into wild-type mice and showed that infiltrating recipient osteoclasts permitted the generation of normal cortical bone in the transplanted tissue. Although these authors suggested that M ϕ s were not involved in resolution of the bone osteoblast function in this model, subsequent studies of the control of osteoblast calcification^{104,105} indicate that the loss of M ϕ s associated with the bone surface probably also contributes to

any bone phenotype. Interestingly, Chitu et al.¹⁰⁶ reported that the *Csf1^{op/op}* is also lethal on a different inbred background (C57BL/6), where more survive on the FVB background and 60–80% of mutant mice survive to weaning on the original mixed genetic background. Another group¹⁰⁷ studied a more limited backcross of the *Csf1^{op/op}* to this genetic background and found that the homozygotes could be maintained to adulthood with careful husbandry and feeding. The idiosyncrasies of C57BL/6 mice as a model for M ϕ biology have been reviewed elsewhere.¹ One relevant feature is that they have an intrinsically low bone density. Female C57BL/6 develop spontaneous osteoporosis at a relatively young age, and this can be blocked by anti-CSF1R treatment.⁸⁸ If anything, the ligand mutation in C57BL/6 mice is more severe than the receptor mutation. Curiously, heterozygous mutation of *Csf1r* abolished the pre-weaning mortality of the *Csf1^{op/op}* mutation.³⁹ There is a similar paradox in the case of *Flt3*, which encodes a receptor tyrosine kinase (FLT3) related to CSF1R. FLT3 and CSF1R are both present on the cell surface of a shared bone marrow progenitor of the monocyte-dendritic cell lineage in mice.¹⁰⁸ Mutation of the ligand gene (*Flt3l*) has an even greater impact than mutation of the receptor gene (*Flt3*) on dendritic cell maturation. Durai et al.¹⁰⁹ proposed that FLT3 is retained on the cell surface in the absence of FLT3L and effectively cross-competes for signaling molecules on progenitor cells with CSF1R. On the other hand, the absence of FLT3 permits increased CSF1R signaling and partial rescue of the dendritic cell deficiency. The potential cross-talk between tyrosine kinase receptors may be relevant to mutant CSF1R phenotypes and variable penetrance in humans discussed below.

Leaving aside whether the differences in apparent severity of ligand and receptor mutations are dependent on genetic background, another explanation for any increased severity of the *Csf1r* mutation relative to the *Csf1^{op/op}* was provided by the identification of the second ligand, IL-34. The knockout of the mouse *Il34* gene (removal of Exons 3–5; inbred C57BL/6 background) revealed a substantial but incomplete depletion of microglia in the brain and loss of Langerhans cells in the skin.¹¹⁰ IL-34 activation of *Csf1r* appears functionally equivalent to CSF1 in that transgenic expression of *Il34* under the control of the *Csf1* promoter rescued the phenotypes of the *Csf1^{op/op}* mouse (at least on an inbred FVB/NJ background).¹¹¹ The impact of the *Il34* mutation is consistent with the major sites of expression of *Il34* in both mice and humans.¹³ Within the mouse brain, *Il34* and *Csf1* have distinct and largely non-overlapping distributions across regions, and the knockouts of the 2 ligands accordingly show distinct impacts on regional microglial densities.¹⁰⁶ Studies in vitro do not support the idea that CSF1 and IL-34 have any differential signaling effects on microglia.¹¹² A second receptor for IL-34, PTPRZ, has been identified¹¹³ but thus far no phenotype has been described in the mouse *Il34* knockout that is incompatible with effects solely mediated by CSF1R. Recent studies have extended the analysis of differential *Il34* dependency to distinct niches in the retina¹¹⁴ and to M ϕ s of the kidney.¹¹⁵ By contrast to mice, extensive profiling of human brain regions in the FANTOM5 project did not indicate a significant excess of *IL34* over *CSF1* mRNA, nor any region specificity.¹³ However, the transcriptomic data do identify separate promoters/transcription

start sites associated with expression of *IL34* in skin and brain in both mouse and human.

As discussed above, some impacts of *Csf1r* mutations on the brain have also been attributed to direct actions on neuronal cells.⁴⁸ Erblich et al.⁴⁷ on the other hand, demonstrated clearly that *Csf1r* mRNA and protein and *Csf1r* reporter genes were expressed exclusively in microglia. They described in detail the progressive postnatal enlargement of the lateral ventricles in *Csf1r^{-/-}* mice on the C57BL/6N background and argued that many of the impacts of the mutation may be secondary to severe hydrocephalus. Consistent with this view, and further highlighting the impact of genetic background, mutations in the genes encoding the junctional adhesion molecule JAM3 and the FYN kinase also produce severe hydrocephalus when crossed to the C57BL/6 background with phenotypic consequences in the brain parenchyma resembling the *Csf1r* knockout.^{116,117}

1.4 | The rat as an alternative rodent model of *Csf1r* deficiency

There have been considerably fewer studies of CSF1R signaling biology in the rat. The toothless rat (*Csf1^{tl/tl}*) has a frame-shift mutation in the *Csf1* gene that ablates function.^{118,119} Most studies of the *Csf1^{tl/tl}* rat have focused on the bone phenotype and the control of tooth eruption. By contrast to the *Csf1^{op/op}* mouse, which retains some osteoclasts and recovers with age, the *Csf1^{tl/tl}* rat has an almost complete loss of osteoclasts, chondrodysplasia and unremitting osteopetrosis that was only partly overcome by postnatal CSF1 administration.^{119–124} Like *Csf1^{op/op}* and *Csf1r^{-/-}* mice, *Csf1^{tl/tl}* rats also exhibited severe postnatal growth retardation, which was associated with deficiencies in the growth-hormone/IGF1 axis.^{123,125} We recently generated a *Csf1r* knockout rat by homologous recombination in embryonic stem cells.⁴⁰ Like the *Csf1^{op/op}* mice, these rats had a complete deficiency of osteoclasts and also lacked bone-associated M ϕ s (osteomacs) that are required for osteoblast maintenance and both endochondral and intramembranous ossification.^{104,105} Despite the lack of osteoclasts, the bone phenotype of mutant rats was distinct from the mouse with much greater preservation of a marrow cavity and no evidence of extramedullary hematopoiesis in the spleen. Unlike *Csf1r^{-/-}* inbred mice, the large majority of mutant rats survived beyond weaning. Figure 2 shows a comparison of the skeletal phenotypes of adult wild-type and mutant rats.

Analysis of female *Csf1r^{-/-}* rats at 11–12 weeks revealed complete loss of specific M ϕ populations, including microglia, Langerhans cells, splenic marginal zone M ϕ s, and peritoneal M ϕ s whereas other M ϕ populations were partly CSF1R-independent. Both sexes are likely to be infertile. The gonads and secondary sexual organs were poorly-developed in both males and females and the differential growth of males versus females was abolished. One phenotype of the *Csf1r^{-/-}* rats, which was not observed in mutant mice, was the complete loss of visceral adipose tissue. Despite the complete absence of microglia in *Csf1r^{-/-}* rats, there was limited evidence of brain pathology aside from mildly enlarged lateral ventricles, thinning of the corpus callosum, and altered differentiation of dopaminergic neurons. Expression profiling

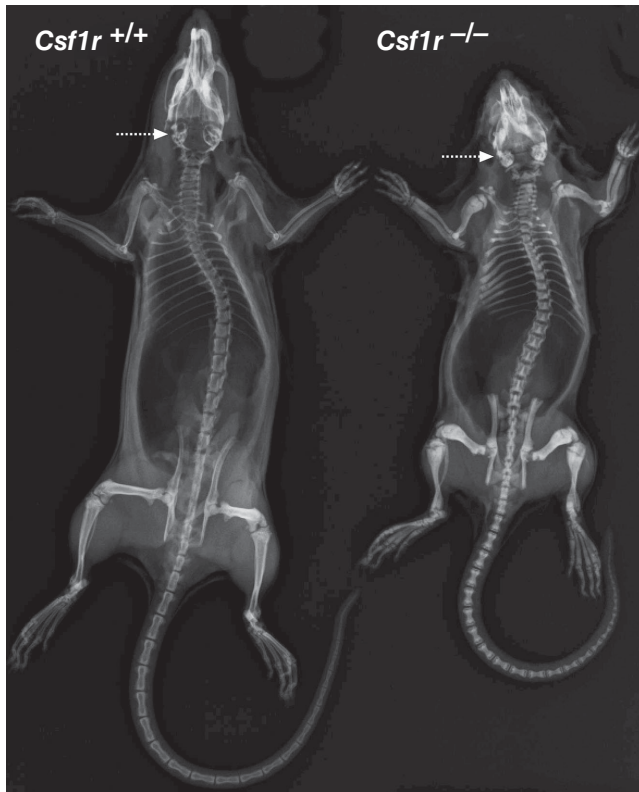


FIGURE 2 The effect of *Csf1r* mutation in rats on skeletal development. The X-ray image compares the skeletal development of an 11-week-old female *Csf1r*^{-/-} rat with a control wild-type littermate.⁴⁰ Note in particular the increased calcification of the skull base (arrows), a phenotype shared with patients with homozygous *CSF1R* mutation.²⁵ There is also an overall reduction in body size and increased calcification of the long bones (especially the hind limbs) and the entire vertebral column, also a feature of the human syndrome²⁵

of several brain regions (hippocampus, olfactory bulb, striatum, pituitary) revealed the selective loss of many known microglia-associated transcripts. However, there was no effect on expression of genes associated with neuronal progenitors (e.g., *Dcx*, *Cux1*); thus providing no support for a non-redundant functional role for *Csf1r* in growth or survival of neuronal progenitor cells.

Since this original study, where the line was an early backcross to an inbred line, we have bred the rat *Csf1r* knockout fully to a fully inbred dark agouti and a fully outbred (Sprague-Dawley) genetic background. On the pure inbred background, survival to weaning is somewhat more compromised and we see more severe ventricular enlargement in the brain and almost complete loss of the olfactory bulbs similar to the *Csf1r*^{-/-} mouse at 3 weeks^{47,48} but in older adult animals (11-12 weeks, unpublished). Regardless of genetic background, the *Csf1r*^{-/-} rats do not exhibit sensory neuronal defects (unlike *Csf1*^{-/-} and *Csf1*^{op/op} mice,³⁹ they are not deaf) nor the loss of Paneth cells in the gut, as reported in mutant mice.

1.5 | Growth deficiency in CSF1R-deficient animals

Mutations of *Csf1r* in both mice³⁹ and rats⁴⁰ lead to reduced body weight. The growth retardation in *Csf1*^{tl/tl} rats was reportedly

associated with an almost complete loss of circulating IGF1.^{123,125} The *Csf1r*^{-/-} rats were indistinguishable from litter mates at birth but in common with *Csf1*^{tl/tl} rats, their growth rate declined rapidly.⁴⁰ The impacts of *Csf1r* mutations have some obvious similarities to growth hormone (GH)/IGF1 mutations (reviewed in ref. 126). Like *Csf1r*^{-/-} animals, GH-deficient (*Gh*^{lit/lit}) or GH receptor-deficient (*Ghr*^{-/-}) mice are born normal size and the growth defect manifests from around 2 weeks of age. *Igf1* deficiency has a greater impact on embryonic growth than *Gh* or *Ghr* mutation but as is the case with *Csf1r* mutation, the perinatal lethality depends on genetic background.¹²⁷ Although the liver is the main source of IGF1 in the circulation, conditional deletion of *Igf1* in hepatocytes did not cause a substantial reduction in postnatal growth.¹²⁶ Mouse Mφs grown in CSF1 also express very high levels of *Igf1* mRNA initiated from a separate promoter from that used in the liver.¹²⁵ Chitu and Stanley⁴⁴ have argued that the growth deficiency in *Csf1* or *Csf1r* mutant animals is secondary to the bone/skeletal abnormality; essentially a consequence of osteoclast deficiency. They noted that transgenic expression of human CSF1 in mouse bone from an osteoblast-specific promoter was able to overcome the growth and skeletal defects in *Csf1*^{op/op} mice.¹²⁸ This finding does not argue against a central role for CSF1 in growth control since CSF1 was also elevated in plasma in these transgenic mice and the impact on circulating IGF1 was not measured. Chang et al.¹²⁹ confirmed the high expression of *Igf1* mRNA in inflammatory Mφs in adipose tissue, but conditional deletion of *Igf1* in myeloid cells using *LysM*-cre did not produce a change in circulating IGF1, growth, or body composition. However, *LysM*-cre does not produce efficient recombination in most resident tissue Mφ populations (see ref. 130). Expression profiling of the liver of *Csf1r*^{-/-} rats revealed a relatively small reduction in *Ghr* and *Igf1*, probably insufficient to explain the growth retardation⁴⁰ and suggesting there is not a primary GH deficiency. There are many other possible axes of regulation that might explain the loss of control of circulating IGF1 by CSF1R-dependent Mφs. IGF1 has a very short plasma half-life unless bound by members of the IGFBP family. Altered expression of these proteins in liver or in other tissues, or changes in their posttranslational modification, could lead to indirect impacts on circulating IGF1.¹³¹ Additionally, recent studies have shown that IGF1 is generated in muscle as a pro-IGF1 form that requires processing.¹³² Conditional deletion of muscle-specific *Igf1* expression can also reduce circulating IGF1 and impair somatic growth¹³³ whereas Mφ-expressed *Igf1* appears essential for muscle regeneration following injury.¹³⁴ As well as contributing directly as a source of IGF1 production, Mφs are obvious candidates for a role in proteolytic processing of both pro-IGF1 and IGFbps. Whereas the precise mechanism is unclear and probably complex, CSF1R mutation clearly impacts the GH-IGF1 axis and many of the pleiotropic consequences in rodents are probably linked to that impact.

1.6 | A hypomorphic *Csf1r* mutation in mice

The transcriptional regulation of the *Csf1r* gene has been analyzed in detail in both mice and humans (reviewed in ref. 20). The major Mφ-specific transcription start site region contains multiple binding sites

for the $M\phi$ -expressed transcription factor, PU.1. The expression of the *Csf1r* reporter genes in $M\phi$ s in transgenic mice depends upon a highly conserved intronic enhancer element (FIRE).³² To test the function of FIRE in its normal genomic context, we deleted the 300 bp sequence from the mouse germ line to produce *Csf1r* ^{Δ FIRE/ Δ FIRE} mice.¹³⁵ Given the level of conservation, we anticipated a phenocopy of the *Csf1r* knockout. Instead, we found a selective loss of certain tissue $M\phi$ populations, notably complete absence of microglia, Langerhans cells of the epidermis, and heart, kidney, and peritoneal $M\phi$ s. However, most major $M\phi$ populations expressed *Csf1r* normally and/or were unaffected in *Csf1r* ^{Δ FIRE/ Δ FIRE} mice; the animals have normal bones, grow normally, and are fertile. Furthermore, the mutation distinguishes the brain-associated and perivascular $M\phi$ populations,^{136,137} which were retained despite their complete loss of *Csf1r* expression, from the microglia, which were lost. Despite the complete loss of microglia, and of *Csf1r* mRNA and other microglial markers in the brain, there was no apparent brain phenotype in *Csf1r* ^{Δ FIRE/ Δ FIRE} mice and no detectable change in expression of any genes that are not clearly myeloid-associated. These mice, along with the *Csf1r* null mutation on outbred genetic backgrounds in mice and rats, tell us that the functions of microglia in development are largely redundant. The elimination of microglia in adults has no obvious pathological impact and seems to protect against several forms of brain injury.^{68,138,139} Similarly, the microglial deficiency in *I134* mutant mice (~80% in most brain regions) has no apparent phenotypic consequences in the steady state but does compromise the ability of mice to deal with pathology associated with viral infection of the brain.¹¹⁰ These findings support the view that the effects of *Csf1r* deletion on the brain may be attributable in part to the primary hydrocephalus, which does not occur in *Csf1r* ^{Δ FIRE/ Δ FIRE} or the *I134*^{-/-} mice and is greatly delayed in *Csf1r*^{-/-} rats. The effects of *Csf1r* inhibitors such as PLX3397⁵¹ discussed above and *Csf1r* knockouts may also depend in part upon systemic effects including the loss of circulating IGF1 described above. For example, we suspect that impacts of the microbiome on microglial numbers and maturation depend indirectly upon peripheral CSF1 deficiency and consequent changes in peripheral $M\phi$ populations.^{140,141}

1.7 | What do the rodent models tell us about the impact of human CSF1R mutations?

ALSP is an adult-onset degenerative disease of the brain leading to multiple cognitive, behavioral, and motor dysfunctions.^{22,23} The histological hallmark of the disease is white matter destruction leading to the loss of axons and myelin, thinning of the corpus callosum, calcification, axonal swellings ('spheroids'), and the accumulation of pigmented $M\phi$ s. The point mutations in CSF1R associated with ALSP (reviewed in refs. 22 and 23, and annotated in Ensembl) are concentrated in the intracellular tyrosine kinase domain. The entire intracellular domain of CSF1R is highly conserved across vertebrate species and even the chicken CSF1R can signal to support proliferation and differentiation when expressed in mammalian cells.¹⁴² Without exception ALSP-associated mutations alter amino acids that are conserved in the chicken receptor. We expressed CSF1R containing a range

of human mutations in the BaF3 factor-dependent cell line where wild-type receptor can sustain CSF1-dependent proliferation.⁶⁵ Whereas each of the mutant proteins was defective in signaling, they were expressed on the cell surface at comparable levels to the wild-type receptor, bound CSF1, and were internalized in response to binding. In an ALSP patient heterozygous for a CSF1R point mutation that abolishes kinase activity, we predict that 75% of receptor dimers will be signaling defective; either homodimers of the mutant protein (25%) or heterodimers with one mutant and one wild-type subunit (50%). These receptor dimers will nevertheless bind and internalize CSF1 thereby competing with the remaining 25% of functional receptor dimers. As discussed above, mutant receptors may also compete for signaling pathways with other receptors such as FLT3 that would otherwise mitigate the impact of CSF1R deficiency. Hence, our data support a dominant negative model for ALSP.

The alternative to a dominant-negative model of disease is to propose that the mutant proteins have no function and reduction of normal peptides to 50% (haploinsufficiency) is enough to cause disease. Neither the animal models nor the human disease patients support this proposal. In rats and mice heterozygous null mutation of *Csf1r* is not dosage compensated. Monocytes and $M\phi$ s in blood and bone marrow of heterozygous mutants express half the level of *Csf1r* mRNA and protein.^{39,40} This is also the case in *Csf1r* ^{Δ FIRE/+} mice.¹³⁵ In the expression profiles of the brains of heterozygous *csf1r*^{+/-} rats and *Csf1r* ^{Δ FIRE/+} mice, there is a 50% reduction in *Csf1r* mRNA. Nevertheless, there is no significant change in any other transcript in response to the 50% loss of *Csf1r*.^{40,135} The lack of dosage compensation is rather surprising since CSF1 can induce down-regulation *Csf1r* mRNA in $M\phi$ s.¹⁴³ One might have anticipated that reduced CSF1R signals would permit up-regulation of expression from the wild-type allele. A further puzzling finding is that despite the 50% reduction in *Csf1r* mRNA and CSF1R protein in heterozygous mutant mice no increase in circulating CSF1 was detected.³⁹ It seems that haploinsufficiency for *Csf1r* has little impact in mice or rats and is unlikely to explain the human dominant disease.

By contrast to the ALSP-associated mutations, the CSF1R mutations described in the recessive syndrome with skeletal symptoms^{25,26} all appear to result in complete or partial loss of function or expression of the protein. Some lead to the generation of premature stop codons and nonsense-mediated mRNA decay, others involve cryptic splice acceptors leading to greatly reduced expression of the wild-type mRNA. Heterozygous carriers are therefore "models" for genuine haploinsufficiency. The recessive disease varies greatly between individuals from infant mortality to adult-onset. Monies et al.¹⁴⁴ described a truncating null mutation (Y540*) in CSF1R in a consanguineous family. The mutation would abolish the intracellular domain including the kinase region and apparently led to early postnatal lethality and severe brain and skeletal phenotypes. These individuals may be the only report of definitive human CSF1R null individuals; their heterozygous parents and siblings were asymptomatic. Guo et al.²⁵ reported another null mutation (Q481*) as one of the compound heterozygous alleles in an individual with recessive skeletal dysplasia and brain disorders. Similar to p.Y540*, p.Q481* would abolish

the intracellular part of the receptor. The RT-PCR analysis for cells derived from this patient demonstrated that mRNA from the mutant allele is subjected to complete NMD. All the heterozygous carriers of Q481* and Y540* in the 2 families were asymptomatic. Guo et al.²⁵ attribute some of the variation in phenotype in the recessive disease to the residual production of wild-type *CSF1R* mRNA (in the case of splice variation) or some retained signaling function of the mutant *CSF1R* encoded by one of the alleles. One disease-associated variant, P132L, present as a compound heterozygote with the definitive loss-of-function allele (Q481*), affects an amino acid in the domain 2 ligand binding domain. While this amino acid is not involved directly in binding either CSF1 or IL34^{27,28} the proline is immediately adjacent to a conserved cysteine involved in the immunoglobulin fold, and is conserved in chickens.¹⁴² Both this mutation, and a mutation in the tyrosine kinase domain (K627del) that was also present as a compound heterozygote in affected individuals, retained some biological activity when expressed in a reporter system.²⁵ Oosterhof et al.²⁶ described a family where affected individuals were homozygous for H643Q mutation in the kinase domain (also a conserved amino acid in chicken) and heterozygous family members were unaffected. They speculate that by contrast to ALSP mutations, the H643Q mutant protein retains some residual kinase activity. This would clearly be testable in the BaF3 cell line system.⁶⁵

A small number of studies have identified loss-of-function mutations in *CSF1R* as the primary causal mutation in ALSP patients and have advocated haploinsufficiency as a mechanism underlying the disease.¹⁴⁵⁻¹⁴⁷ The proposal is supported by ALSP-like symptoms developing in a subset of aged male heterozygous *Csf1r* mutant mice on the C57BL/6 background,¹⁴⁸ a background on which the homozygous mutant is lethal prior to weaning. But in this model, there was a substantial increase in microglial numbers throughout the brain compared to wild-type mice. A detailed analysis of the brains of multiple ALSP patients revealed the direct reverse of the mouse phenotype.¹⁴⁹ By contrast to other leukodystrophies, in which microglia appear increased and activated, there were greatly reduced microglial numbers, uneven distribution, and focal changes in morphology referred to as dysplasia. The loss of microglia was associated with a marked loss of detectable *CSF1R* protein and the microglial marker CD11b on Western blots in clear contrast to increased detection in other disease states exhibiting microgliosis. In ALSP patients with heterozygous loss-of-function mutations and a diagnosis of ALSP the expression of the wild-type allele at the protein level in microglial progenitors is not known. The lack of any evidence of disease in family members with heterozygous complete loss of function alleles of *CSF1R* associated with the recessive disease^{25,26} strongly supports the view that haploinsufficiency per se is not likely to explain the pathology of ALSP.

Although coding variations in the intracellular domain of *CSF1R* are comparatively rare, examination of the ExAC database (exac.broadinstitute.org) reveals several (T672H, A629S, T621M, T600M, T587I, R549H, R549C) detected in multiple individuals that affect amino acids in the intracellular kinase domain that are conserved in birds. By inference these mutations are likely to com-

promise receptor activity. If that is the case, ALSP may be the tip of a less penetrant iceberg of *CSF1R*-dependent microglial dysfunction (likely diagnosed as other disease entities in the absence of *CSF1R* genotyping or autopsy^{22,150}) or alternatively, these variants might indeed produce complete loss of function but cause disease only in homozygotes. Coding variants are considerably more common in the extracellular domain but none affects contact amino acids involved directly in ligand binding. However, Yeh et al.¹⁵¹ recently described a *CSF1R* variant (H362R) with a high-allele frequency in the East Asian population. They presented evidence that this change within the receptor dimerization domain partly compromises signaling, but others found that the variant did not prevent autophosphorylation.¹⁴⁷

One of the two recent reports of the recessive disease²⁶ promoted the zebrafish as an alternative model of *Csf1r* deficiency, in part because the mutation in inbred mice is apparently much more severe than the human disease. It is certainly the case that the function of *Csf1r* in the generation of *Mφs* and microglia is conserved in fish^{16,17,152} as it is in birds that provide an alternative tractable model in which development can be monitored in ovo.¹⁵ We have produced a *Csf1r* deletion in chickens and observed the same severe growth retardation (post-hatch) seen in mice and rats (DAH, A. Balic; unpublished). One complexity of working with zebrafish, other than the quite distinct skeletal and hematopoietic biology, is that much of the genome is duplicated and there are two *Csf1r* loci with partially redundant functions. The generation of an allelic series with graded loss of 1 to 4 copies of *Csf1r* indicated that microglial numbers are sensitive to *Csf1r* dosage and further, that as in mice and rats (see above) some peripheral *Mφ* populations were less dependent upon *Csf1r*.¹⁵² However, as noted above, 50% loss of *Csf1r* in the brain of rats and mice does not compromise microglial numbers. The phenotypes of mouse and rat *Csf1r* mutations on inbred and outbred backgrounds show the same range of phenotypes as observed in human patients and provide an informative and relevant model to test therapies such as bone marrow transplantation. The clear impact of genetic background implies the existence of epistatic modifiers of disease phenotype, likely providing an additional explanation for the diversity of age-of-onset. Indeed, one might consider the possibility that consanguineous couples could also generate homozygosity for epistatic modifiers of the impact of *CSF1R* mutations in their affected progeny.

Adult-onset patients with homozygous *CSF1R* mutations have not been reported to exhibit the severe postnatal growth retardation seen in mice and rats.²⁵ We suggest that the impact of mutation on the GH/IGF1 axis requires the complex loss of *CSF1*-*CSF1R* activity and peripheral *Mφ* populations and it may have contributed to the more severe cases with infant mortality. In any case, the absence of this phenotypic impact supports the argument above that osteopetrosis per se is insufficient explanation for growth retardation. Otherwise the differences from mutant animal phenotypes are not that great. It is clear from the mouse and rat mutations of *Csf1r* that microglia and osteoclasts are *CSF1R*-dependent. Other populations that share *CSF1R*-dependence with microglia, such as Langerhans cells, peritoneal *Mφs*, and heart and kidney *Mφs*, have not been studied in either ALSP or homozygous/compound heterozygous mutant patients. ALSP patients,

who certainly do have residual CSF1R activity, do not exhibit the skeletal phenotypes described in the recessive disease but they do show ectopic calcification in the brain.¹⁴⁶ As discussed above, the osteoclast deficiency in outbred mouse *Csf1r*^{-/-} mice resolves with age, indicating that there are other factors that can compensate for the loss of CSF1R. Similarly, our *Csf1r*^{ΔFIRE/ΔFIRE} mice lack microglia but they have normal Mφs and osteoclasts in the bone despite the lack of *Csf1r* expression in those cells. We can conclude therefore that microglia are likely more sensitive than osteoclasts to disruption of CSF1R signals. In simple terms, we suggest that the autosomal dominant and recessive diseases associated with *CSF1R* mutations are part of the same spectrum of CSF1R insufficiency, with ALSP patients retaining enough CSF1R activity to maintain osteoclasts and most tissue Mφ populations. That said, the impact of the ALSP-associated mutations on peripheral Mφ biology has not been adequately considered in dissecting disease mechanisms. Several recent studies have emphasized the relationship between resident tissue Mφs and the peripheral nervous system (e.g., ref. 153) and we have identified very large numbers of Mφs in smooth and skeletal muscle¹; loss of function of these cells could potentially contribute to motor defects in ALSP patients.¹ Kupffer cells were apparently unaffected in the livers of ALSP patients¹⁴⁹ but they are also relatively CSF1R-independent in animals.⁴⁰ There is clearly some impact of ALSP mutations in the periphery, since there is a defect in the CSF1-dependent generation of so-called non-classical monocytes in patients.¹⁵⁴ So, further studies of peripheral Mφ populations in ALSP patients are needed in order to fully understand the disease process.

2 | CONCLUSIONS

In overview, we suggest that mouse and rat mutations provide informative and predictive models of the pathology of human *CSF1R* deficiency provided account is taken of genetic background and the variable sensitivity of different Mφ populations to CSF1R loss of function. The evidence for functional expression of *CSF1R* in non-hematopoietic cells is not compelling and accordingly all of the phenotypes associated with mutation of *CSF1R* or its ligands can be attributed to their impacts on mononuclear phagocyte biology. In keeping with that conclusion, all of the pleiotropic impacts of a *Csf1r* mutation in mice can be overcome by neonatal bone marrow transplantation.^{155,156} The rat *Csf1r*^{-/-} model with improved postnatal viability offers the opportunity to test therapies that might reverse the adverse phenotypes of human *CSF1R* mutations later in postnatal development or even in adults. Transplantation and other interventions proven efficacious in mouse and rat models are likely to provide insight into the human condition and may offer promise to patients with these rare diseases.

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All authors contributed to writing and editing of the manuscript.

ORCID

David A. Hume  <https://orcid.org/0000-0002-2615-1478>

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