

# UC Irvine

## UC Irvine Previously Published Works

### Title

MAC2 is a long-lasting marker of peripheral cell infiltrates into the mouse CNS after bone marrow transplantation and coronavirus infection

### Permalink

<https://escholarship.org/uc/item/4xh823kz>

### Journal

Glia, 70(5)

### ISSN

0894-1491

### Authors

Hohsfield, Lindsay A  
Tsourmas, Kate I  
Ghorbanian, Yasamine  
et al.

### Publication Date

2022-05-01




### DOI

10.1002/glia.24144

Peer reviewed

## RESEARCH ARTICLE

# MAC2 is a long-lasting marker of peripheral cell infiltrates into the mouse CNS after bone marrow transplantation and coronavirus infection

Lindsay A. Hohsfield<sup>1</sup> | Kate I. Tsourmas<sup>1</sup> | Yasamine Ghorbanian<sup>2,3</sup> |  
Amber R. Syage<sup>1</sup> | Sung Jin Kim<sup>1</sup> | Yuting Cheng<sup>1</sup> | Susana Furman<sup>1</sup> |  
Matthew A. Inlay<sup>2,3</sup>  | Thomas E. Lane<sup>1</sup>  | Kim N. Green<sup>1</sup> 

<sup>1</sup>Department of Neurobiology and Behavior, University of California, Irvine, Irvine, California, USA

<sup>2</sup>Sue and Bill Gross Stem Cell Research Center, University of California, Irvine, Irvine, California, USA

<sup>3</sup>Department of Molecular Biology and Biochemistry, University of California, Irvine, Irvine, California, USA

## Correspondence

Kim N. Green, 3208 Biological Sciences III, University of California, Irvine, Irvine, CA 92697-4545, USA.  
Email: kngreen@uci.edu

Thomas E. Lane, Department of Neurobiology & Behavior, School of Biological Sciences, University of California, Irvine, CA, USA.  
Email: tlane@uci.edu

## Funding information

Alzheimer's Association, Grant/Award Number: AARF-16-442762; National Institutes of Health, Grant/Award Number: NS082174; National Institutes of Health/National Institute of Aging, Grant/Award Numbers: RF1AG056768, RF1AG065329, U54AG054349; National Institutes of Health/National Institute of Neurological Disorders and Stroke, Grant/Award Numbers: R01NS041249, R01NS083801, R35NS116835; National Multiple Sclerosis Society, Grant/Award Number: CA-1607-25040; The Ray and Tye Noorda Foundation

## Abstract

Microglia are the primary resident myeloid cells of the brain responsible for maintaining homeostasis and protecting the central nervous system (CNS) from damage and infection. Monocytes and monocyte-derived macrophages arising from the periphery have also been implicated in CNS pathologies, however, distinguishing between different myeloid cell populations in the CNS has been difficult. Here, we set out to develop a reliable histological marker that can assess distinct myeloid cell heterogeneity and functional contributions, particularly in the context of disease and/or neuroinflammation. scRNAseq from brains of mice infected with the neurotropic JHM strain of the mouse hepatitis virus (JHMV), a mouse coronavirus, revealed that *Lgals3* is highly upregulated in monocyte and macrophage populations, but not in microglia. Subsequent immunostaining for galectin-3 (encoded by *Lgals3*), also referred to as MAC2, highlighted the high expression levels of MAC2 protein in infiltrating myeloid cells in JHMV-infected and bone marrow (BM) chimeric mice, in stark contrast to microglia, which expressed little to no staining in these models. Expression of MAC2 was found even 6–10 months following BM-derived cell infiltration into the CNS. We also demonstrate that MAC2 is not a specific label for plaque-associated microglia in the 5xFAD mouse model, but only appears in a distinct subset of these cells in the presence of JHMV infection or during aging. Our data suggest that MAC2 can serve as a reliable and long-lasting histological marker for monocyte/macrophages in the brain, identifying an accessible approach to distinguishing resident microglia from infiltrating cells in the CNS under certain conditions.

## KEYWORDS

Alzheimer's disease, brain, *Lgals3*, MAC2, microglia, monocytes, mouse coronavirus

## 1 | INTRODUCTION

Myeloid cells of the innate immune system are comprised of two distinct populations: tissue-resident and monocyte-derived macrophages. In the central nervous system (CNS), the macrophage compartment consists of

parenchymal macrophages, known as microglia, and non-parenchymal macrophages, known as CNS (or border)-associated macrophages (CAMs or BAMs) (Kierdorf et al., 2019; Mrdjen et al., 2018). Microglia reside in the brain parenchyma spatially arranged in discrete non-overlapping territories, while BAMs reside within specialized CNS border interfaces that

separate the CNS from the periphery, including the choroid plexus, meninges, and perivascular spaces. Recent studies indicate that microglia and non-parenchymal macrophages represent ontologically, phenotypically, and functionally distinct cell populations (Jordao et al., 2019; Masuda et al., 2019; Mrdjen et al., 2018; Munro et al., 2020; Shemer et al., 2018; Utz et al., 2020; Van Hove et al., 2019); however, inflammation-induced phenotypic changes in these cells have made distinguishing and assessing different myeloid cell populations and their diverse functions during disease difficult.

In mice, studies have shown that microglia derive from erythromyeloid precursors in the yolk sac, entering the brain as primitive or pre-macrophages to colonize the CNS during embryonic development (Ginhoux et al., 2010; Kierdorf et al., 2013). Once the blood–brain barrier (BBB) is formed, myeloid cell infiltration into the CNS is restricted and microglial population maintenance relies on local self-proliferation without contributions from peripheral monocytes (Shemer et al., 2015). While studies have shown that specific BAM subsets (e.g. perivascular and leptomeningeal macrophages) also derive from the yolk sac (Gomez Perdiguero et al., 2015; Utz et al., 2020), it appears that choroid plexus and dural macrophage populations can be replenished by bone marrow (BM)-derived monocytes under steady-state conditions (Goldmann et al., 2016; Van Hove et al., 2019). Under inflammatory or disease conditions (e.g., facial nerve axotomy, amyotrophic lateral sclerosis, and experimental autoimmune encephalomyelitis), studies have shown that peripheral-derived monocytes enter the brain, but do not contribute significantly to the microglial pool unless under defined experimental circumstances (e.g., irradiation, myeloablative chemotherapy, and BBB disruption) (Ajami et al., 2007; Ajami et al., 2011; Cronk et al., 2018; Ginhoux et al., 2010; Hohsfield et al., 2020; Jordao et al., 2019; Lund et al., 2018; Mildner et al., 2007; Reed-Geaghan et al., 2020).

Despite their distinct origins and functions, assessing the individual contributions of infiltrated myeloid cells and activated microglia remains one of the major challenges in neuroimmunology, particularly within the context of neuroinflammatory diseases (Herz et al., 2017; Honarpisheh et al., 2020; Prinz & Priller, 2014). Monocytes and microglia share substantial transcriptional and phenotypic overlap. Several of the genes and proteins used to separate these two populations are not exclusively expressed but instead only relatively enriched in one population (e.g., CD45, CX3CR1, and CCR2) (Haage et al., 2019; Honarpisheh et al., 2020; Ransohoff & Cardona, 2010; Shemer et al., 2015). Microglial-specific genes and proteins have been identified; however, microglia downregulate their homeostatic signature (e.g., *P2ry12*, *Tmem119*, *Sall1*, and *Siglech*) and upregulate monocyte/macrophage-associated (e.g., *Apoe*, *Itgax*, and *Lyz2*) markers during disease (Bennett et al., 2016; Butovsky et al., 2014; Buttgerit et al., 2016; Keren-Shaul et al., 2017; Konishi et al., 2017). Studies have also identified genes and proteins that are highly expressed in BM-derived myeloid cells, including brain engrafted macrophages (e.g., *Ms4a7*, *Clec12a*, *Ccr2*, *Apoe*, *CD38*, *Mrc1*, and *Cd11a*), but many of these genes are also found in other CNS myeloid subsets or fail to label cells during immunohistochemical investigations on fixed brain sections (Bennett et al., 2018; Mrdjen et al., 2018; Shemer et al., 2018; Shukla et al., 2019). As such, there is no histological marker that can reliably identify peripheral myeloid cell infiltrates versus activated microglia in the CNS to date.

Researchers currently utilize a number of experimental approaches to identify peripheral myeloid cells in the CNS. However, many of these paradigms pose concerning caveats. The generation of GFP BM chimeras provides labeling of BM-derived cells, but this approach involves irradiation, which can induce BBB damage and non-homeostatic peripheral contributions to the microglial niche (Ajami et al., 2007; Mildner et al., 2007). Parabiosis avoids these irradiation-induced effects but can involve complicated surgery and result in host-graft rejection. Despite the usefulness of mice genetically labeled for monocyte markers (e.g., *Ccr2*<sup>RFP</sup> mice), studies have shown that these cells may downregulate CCR2 as they mature/differentiate and enter the brain (Gschwandtner et al., 2019; Saederup et al., 2010). Furthermore, these mice are haploinsufficient for CCR2, a critical factor for monocyte migration and entry into the CNS (Chu et al., 2014). Fate mapping techniques have allowed for unprecedented exploration and manipulation of monocytes, but again these techniques are not without their limitations (Chen, Sun, et al., 2020; Liu et al., 2019). The use of lineage tracing from BM-expressed drivers involves the use of tamoxifen, which has recently been identified as a neutral agonist in macrophage lineage tracing studies. A recent study has shown that tamoxifen treatment of pregnant mice induces a significant expansion of the embryonic macrophage population (Rojo et al., 2018).

Given the lack of reliable markers that can distinguish between monocyte-derived cells and activated microglia, we set out to develop a new histological tool for peripheral myeloid cell studies in the mouse CNS. To accomplish this, we employed infection of C57BL/6 mice with the neuroadapted JHM strain of mouse hepatitis virus (JHMV), a mouse coronavirus and a robust mouse model for monocyte infiltration in the CNS. Studies indicate that JHMV-infected mice exhibit substantial infiltration by monocytes along with other peripheral immune cells (Held et al., 2004; Savarin et al., 2010). We recently employed single-cell RNA sequencing (scRNAseq) on flow-sorted CD45<sup>+</sup> cells infiltrating into the CNS of JHMV-infected mice to better understand the immunological landscape at defined times following infection with a neurotropic virus (Syage et al., 2020). Using this approach, we identified *Lgals3* as a highly enriched gene for monocytes. We then validated the expression of this marker at the protein level via galectin-3/MAC2 staining in BM-derived myeloid cells, and its utility in distinguishing between monocyte and microglia populations in response to JHMV infection as well as in young transgenic mouse models of Alzheimer's disease (AD). This approach not only provides a reliable marker for peripheral-derived cells, but also eliminates the necessity of complex transgenic mouse lines to discern myeloid cell populations, broadening the study of myeloid cell spatial and phenotypic profiles during disease and neuroinflammation.

## 2 | MATERIALS AND METHODS

### 2.1 | Compounds

Pexidartinib (PLX3397) was provided by Plexxikon Inc. and formulated in AIN-76A standard chow at a dose of 600 ppm by Research Diets Inc.

## 2.2 | Mice

All mice were obtained from The Jackson Laboratory. For transplant studies, bone marrow cells were isolated from CAG-EGFP mice (Stock No. 006567). Male and female 5xFAD mice were obtained from MMRRC-JAX (Stock No. 34848) and have been previously described in detail (Oakley et al., 2006). For 5xFAD genotyping, the primer sequences used were PS1 Forward 5'-AAT AGA GAA CGG CAG GAG CA-3' and PS1 Reverse 5'-GCC ATG AGG GCA CTA ATC AT-3'. All other mice were male C57BL/6 (000664) mice. Animals were housed with open access to food and water under 12 h/12 h light-dark cycles.

## 2.3 | Animal treatments

All rodent experiments were performed in accordance with animal protocols approved by the Institutional Animal Care and Use Committee at the University of California, Irvine (UCI). **JHMV infection:** Six-week-old C57BL/6 mice (males and females) and four-month-old 5xFAD mice (males and females) were infected intracranially (i.c.) with 200–250 PFU of JHMV in 30  $\mu$ L of sterile Hanks balanced sterile solution (HBSS); control mice received an i.c. injection of HBSS. JHMV-infected C57BL/6 mice were sacrificed at 7, 14, and 21 days post-infection (d.p.i.) and brains and spinal cords isolated to assess viral titers and perform immunohistochemical staining to evaluate monocyte infiltration. JHMV-infected 5xFAD mice were sacrificed at 10 d.p.i. and brains removed to determine viral titers, evaluate  $\beta$ -amyloid (A $\beta$ ) burden, and perform immunohistochemical staining to assess monocyte infiltration. scRNAseq was conducted on male animals. **Microglial depletion:** Mice were administered ad libitum with PLX3397 at a dosage of 600 ppm (to eliminate microglia) or vehicle (control) for 14d. **Bone marrow transplant:** Tissue from a previous study by Hohsfield et. al were employed for analyses performed in the present report (Hohsfield et al., 2020). In brief, C57BL/6 mice were anesthetized with isoflurane and then irradiated with 1000 cGy (whole body) and reconstituted via retroorbital injection with  $2 \times 10^6$  whole BM cells from CAG-EGFP mice. Blood was measured at 4, 8, and/or 12 weeks post transplantation to track granulocyte chimerism. At time of sacrifice, mice were euthanized, and BM was harvested and analyzed by flow cytometry for HSC chimerism. This established an average percent chimerism of >95% in whole body irradiated mice as previously reported (Hohsfield et al., 2020). **Tissue collection:** Following treatments, adult mice were sacrificed via carbon dioxide inhalation and perfused transcardially with 1X PBS. Brains were extracted and dissected down the midline, with one half flash-frozen for subsequent RNA and protein analyses, and the other half drop-fixed in 4% paraformaldehyde. Fixed brains were cryopreserved in PBS + 0.05% sodium azide + 30% sucrose, frozen, and sectioned at 40  $\mu$ m on a Leica SM2000 R sliding microtome for subsequent immunohistochemical analyses. For brains and spinal cords from JHMV-infected mice, half brains dissected down the midline and the length of spinal cord extending from thoracic vertebra 6–10 was cryoprotected in 30% sucrose, cut into 1-mm transverse blocks and processed to

preserve the craniocaudal orientation and subsequently embedded in O.C.T. (VWR, Radnor, PA, USA). For spinal cords, 8  $\mu$ m-thick coronal sections were cut and sections were stained following standard immunofluorescence protocols; for brains, 8  $\mu$ m sagittal sections were cut (Blanc et al., 2015; Dickey et al., 2016; Marro et al., 2016).

## 2.4 | Histology and confocal microscopy

Fluorescent immunolabeling followed a standard indirect technique as described previously (Elmore et al., 2014). Brain sections were stained with antibodies against the following defined antigens: ionized calcium binding adaptor molecule 1 (IBA1; 1:1000; 019-19,741, Wako and ab5076, Abcam) and MAC2/Galectin-3 (1:500; CL8942AP Cedarlane). For DAPI staining, mounted brain sections were coverslipped using Fluoromount-G with DAPI (00-4959-52, Invitrogen). Amylo-Glo (TR-300-AG; Biosensis) staining was performed according to the manufacturer's instructions. High resolution fluorescent images were obtained using a Leica TCS SPE-II confocal microscope and LAS-X software. For confocal imaging, one field of view (FOV) per brain region was captured per mouse unless otherwise indicated. For whole brain stitches, automated slide scanning was performed using a Zeiss AxioScan.Z1 equipped with a Colibri camera and Zen AxioScan 2.3 software. Microglial morphology was determined using the Filaments module in Bitplane Imaris 7.5, as described previously (Elmore et al., 2015). Cell quantities were determined using the Spots module in Imaris. MAC1+ IBA1+ staining was quantified by using a standardized algorithm in the Surfaces module in Imaris. Total IBA1+ and MAC2+ IBA1+ surface area in the field of view was categorized as plaque-associated or non-plaque-associated based on proximity to plaques (plaque-associated  $\leq 5.8 \mu$ m). Percentage of MAC2+ IBA1+ coverage was calculated by dividing MAC2+ IBA1+/total IBA1+ surface area.

## 2.5 | Data analysis and statistics

Statistical analysis was performed with Prism Graph Pad (v.8.0.1). To compare two groups, the unpaired Student's t-test was used. To compare multiple groups, a one-way ANOVA with Tukey's posthoc test was performed. For all analyses, statistical significance was accepted at  $p < .05$ . All bar graphs are represented as means  $\pm$  SEM and significance expressed as follows: \* $p < .05$ , \*\* $p < .01$ , \*\*\* $p < .001$ .  $n$  is given as the number of mice within each group, unless otherwise indicated.

# 3 | RESULTS

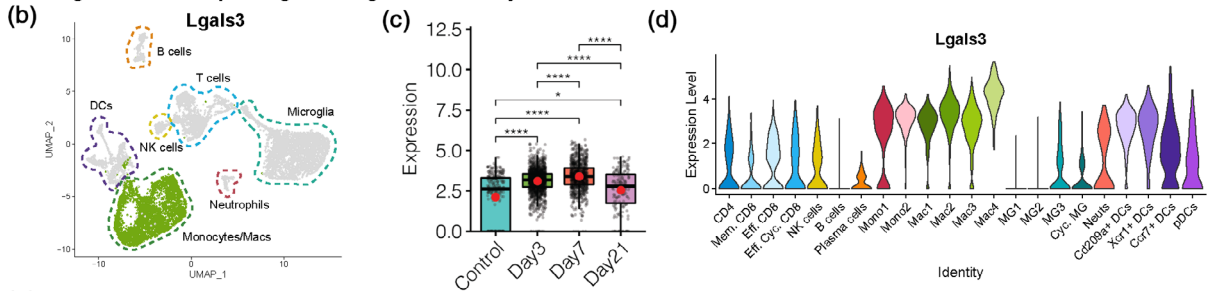
## 3.1 | *Lgals3*/MAC2 distinguishes infiltrating cells from microglia in the adult CNS

We and others have previously identified distinct transcriptional and phenotypic profiles of brain-engrafted peripherally derived myeloid

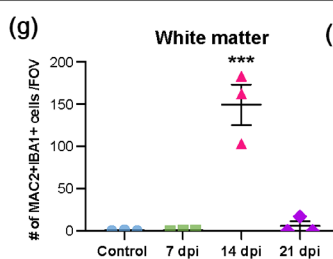
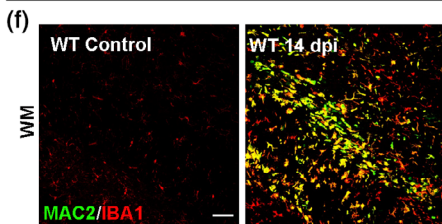
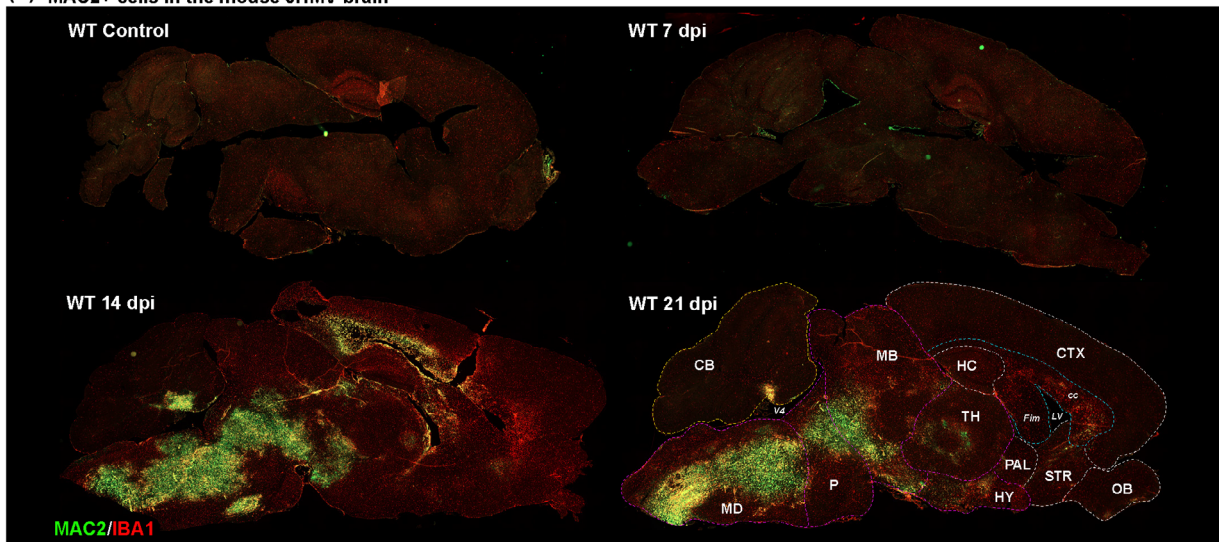
(a) Time course of CNS JHMV infection



Single Cell RNA-seq distinguishes Lgals3 as monocyte marker



(e) MAC2+ cells in the mouse JHMV brain



(h) MAC2+ distribution in JHMV mouse brain

Brain region	Animal group			
	Control	7 dpi	14 dpi	21 dpi
Brainstem	-	-	+++	+++
Cerebellum	-	-	++	+
White matter	-	-	+++	+
Cerebrum	-	-	-	-

(i) MAC2+ cells in the mouse spinal cord

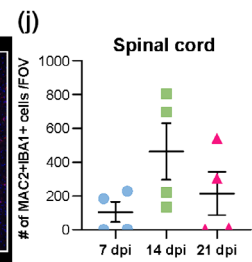
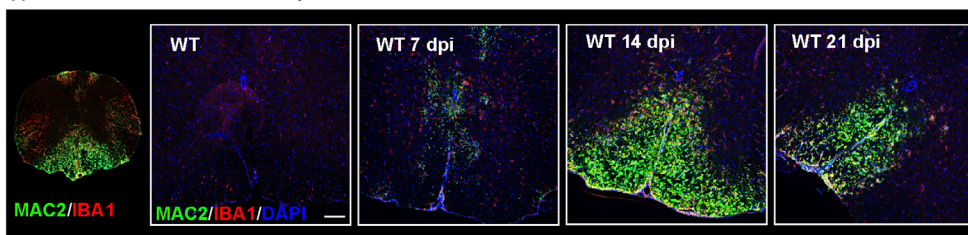


FIGURE 1 Legend on next page.

cells from resident microglia in the adult mouse brain; however, these findings have yet to translate into reliable histological markers that distinguish peripheral infiltrates from their microglial counterparts in the CNS (Bennett et al., 2018; Cronk et al., 2018; Hohsfield et al., 2020; Anat Shemer et al., 2018). In this study, we explored a previously described scRNAseq data set to identify transcripts that distinguish between resident microglia and infiltrating myeloid cells (Syage et al., 2020). scRNAseq data was obtained from the JHMV mouse model which recapitulates many characteristics of human diseases associated with encephalomyelitis and demyelination (Bergmann et al., 2006; Lampert et al., 1973; Lane & Buchmeier, 1997; Savarin et al., 2010; Weiner, 1973). In brief, intracranial inoculation of susceptible C57BL/6 mice with the neuroadapted JHMV, a member of the Coronaviridae family, results in an acute encephalomyelitis characterized by wide-spread viral replication in astrocytes, microglia, and oligodendroglia with relative sparing of neurons (Bergmann et al., 2006; Lane & Hosking, 2010). In response to JHMV infection, proinflammatory cytokines and chemokines are expressed by activated microglia and astrocytes, resulting in an orchestrated inflammatory response consisting of neutrophils, monocytes/macrophages, and activated virus-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cells (Bergmann et al., 2006; Hosking & Lane, 2010; Lane & Hosking, 2010).

We recently performed a detailed analysis of immune cell infiltration into the CNS of JHMV-infected mice through scRNAseq, which highlighted the heterogeneity of the immune response following infection with a neurotropic virus, in addition to confirming that monocytes and neutrophils are the prominent cells entering the CNS (Syage et al., 2020). Here, we employed the scRNAseq data set to identify differentially expressed genes between monocyte/macrophage cells compared to microglia. *Lgals3* transcripts were enriched in infiltrating monocytes and macrophages, and lowly expressed in microglia (specifically in MG3 and Cyc. MG clusters) even under JHMV/inflammatory conditions (Figure 1a–d). *Lgals3* encodes galectin-3 (also known as MAC2), which is a member of the lectin family involved in monocyte/macrophage chemoattraction and activation (MacKinnon et al., 2008). Since low levels of

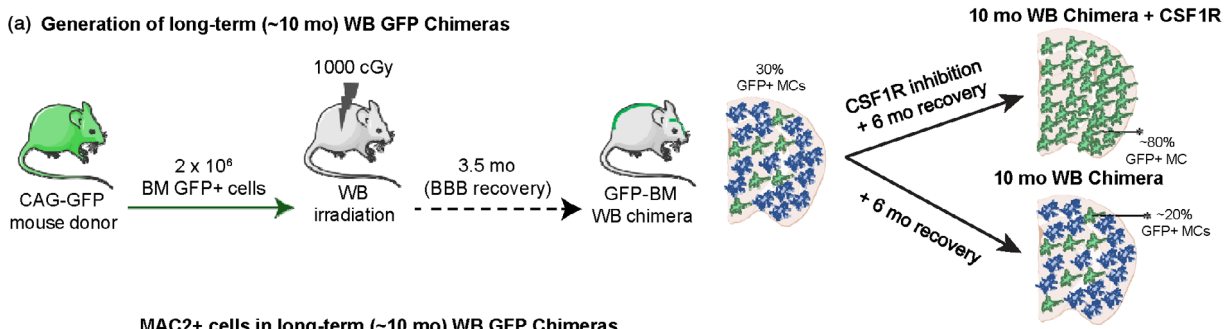
*Lgals3* transcripts were detected in some microglia clusters, we next evaluated whether galectin-3/MAC2 expression was present in both monocyte/macrophage and distinct microglial populations. To assess this, we subsequently infected a separate cohort of C57BL/6 mice with JHMV and evaluated the ability of galectin-3/MAC2 to distinguish distinct myeloid cell populations. Histological analyses for MAC2 in non-infected brain sections revealed no MAC2 staining in IBA1<sup>+</sup> cells, except in areas associated with ventricles and the choroid plexus (Figure 1e and f). Analysis of JHMV-infected brain and spinal cord sections showed a clear population of IBA1<sup>+</sup>/MAC2<sup>+</sup> cells that were not present in uninfected animals. IBA1<sup>+</sup>/MAC2<sup>+</sup> cells appear spatially and morphologically distinct from IBA1<sup>+</sup>/MAC2<sup>-</sup> cells, which resemble microglia and reside in parenchymal areas (Figure 1e, f, and i). In the brain, IBA1<sup>+</sup>/MAC2<sup>+</sup> cells accumulated in the brainstem (medulla, pons, midbrain, thalamus, and hypothalamus), cerebellum (near the fourth ventricle), and in white matter tracts (corpus callosum), whereas little to no deposition was observed in the cerebrum at any timepoint (hippocampus, cortex, and striatum) with the exception of sites near the lateral ventricle and/or white matter tracts (Figure 1e, f, and h). Many areas of high MAC2<sup>+</sup> cell accumulation appear to be in or near white matter areas (Figure 1f). IBA1<sup>+</sup>/MAC2<sup>+</sup> cells appear less ramified with retracted/thickened processes and enlarged cell somas compared to IBA1<sup>+</sup>/MAC2<sup>-</sup> cells (Figure 1f). IBA1<sup>+</sup>/MAC2<sup>+</sup> cell infiltration in the brain appears to peak around 14 d.p.i (Figure 1e, g, and h); however, the brainstem still shows prominent accumulation at 21 d.p.i. In the spinal cord, IBA1<sup>+</sup>/MAC2<sup>+</sup> cell infiltration peaked at 14 d.p.i. and declined by 21 d.p.i. with cells enriched in white matter tracts within the ventral funiculus (Figure 1i and j).

### 3.2 | MAC2 is a specific and long-lasting marker for bone marrow-derived infiltrating cells

Beyond acute CNS infiltration, current monocyte markers make it difficult to distinguish between peripheral and resident myeloid cells.

**FIGURE 1** *Lgals3*/MAC2 distinguishes infiltrating cells from microglia in the adult CNS. (a) Schematic of experimental design of intracranial (i.c.) infection of five to six-week-old C57BL/6 wild type (WT) mice with 250 PFU of the neurotropic JHM-J2.2v-1 strain of mouse hepatitis virus (JHMV). Brains and spinal cords were collected at 7, 14, 21 days post infection (d.p.i.). (b) UMAP plot displays aggregate data of CD45<sup>+</sup> cells and shows scaled expression of transcripts encoding *Lgals3* in monocyte/macrophage (Monocytes/Macs) clusters (Syage et al., 2020). (c) Temporal analysis of expression of transcripts encoding *Lgals3*. Transcript levels were elevated at defined timepoints during JHMV infection. Data is presented as normalized expression values, and random noise was added to show data point distribution. Box plots show interquartile range and median value (bold horizontal bar). Red dots indicate the average expression value per sample. Wilcoxon's test was used for statistical analysis. (d) Violin plot shows gene expression of *Lgals3* in all CD45<sup>+</sup> cells on the single-cell level and that *Lgals3* is upregulated in monocytes and macrophage clusters. (e) Representative whole sagittal brain sections of MAC2 (green) and IBA1 (red) staining in WT non-infected and JHMV-infected mice at 7, 14, and 21 d.p.i. (f) High resolution 20× confocal images of MAC2<sup>+</sup> (green) and IBA1<sup>+</sup> (red) cells in the white matter tracts of WT non-infected and JHMV-infected at 14 d.p.i. (g) Quantification of MAC<sup>+</sup> IBA1<sup>+</sup> cells per field of view (FOV) in white matter tracts of brains in WT control and JHMV-infected mice. (h) Table of the relative MAC2<sup>+</sup> cell distribution in distinct brain regions. – indicates no accumulation. + indicates low accumulation. ++ indicates moderate accumulation. +++ indicates extensive accumulation. (i) Representative confocal images of MAC2<sup>+</sup> (green) and IBA1<sup>+</sup> (red) cells in the spinal cords of WT control and JHMV-infected mice at various timepoints post infection. (j) Quantification of (i). Mouse, syringe and virus illustrations were obtained from Servier Medical Art at smart.servier.com. IBA1, ionized calcium adapter molecule 1; CB, cerebellum; cc, corpus callosum; CTX, cortex; Fim, fimbria; HC, hippocampus; HY, hypothalamus; LV, lateral ventricle; MB, midbrain; MD, medulla; OB, olfactory bulb; P, pons; PAL, pallidum; STR, striatum; TH, thalamus; V4, fourth ventricle. Data are represented as mean ± SEM (n = 3–4 mice/timepoint). \* p < .05, \*\*\* p < .001, \*\*\*\* p < .0001, compared to control unless otherwise indicated. Scale bar in (f) ~100 μm; in (i) inserts ~160 μm

(a) Generation of long-term (~10 mo) WB GFP Chimeras



MAC2+ cells in long-term (~10 mo) WB GFP Chimeras

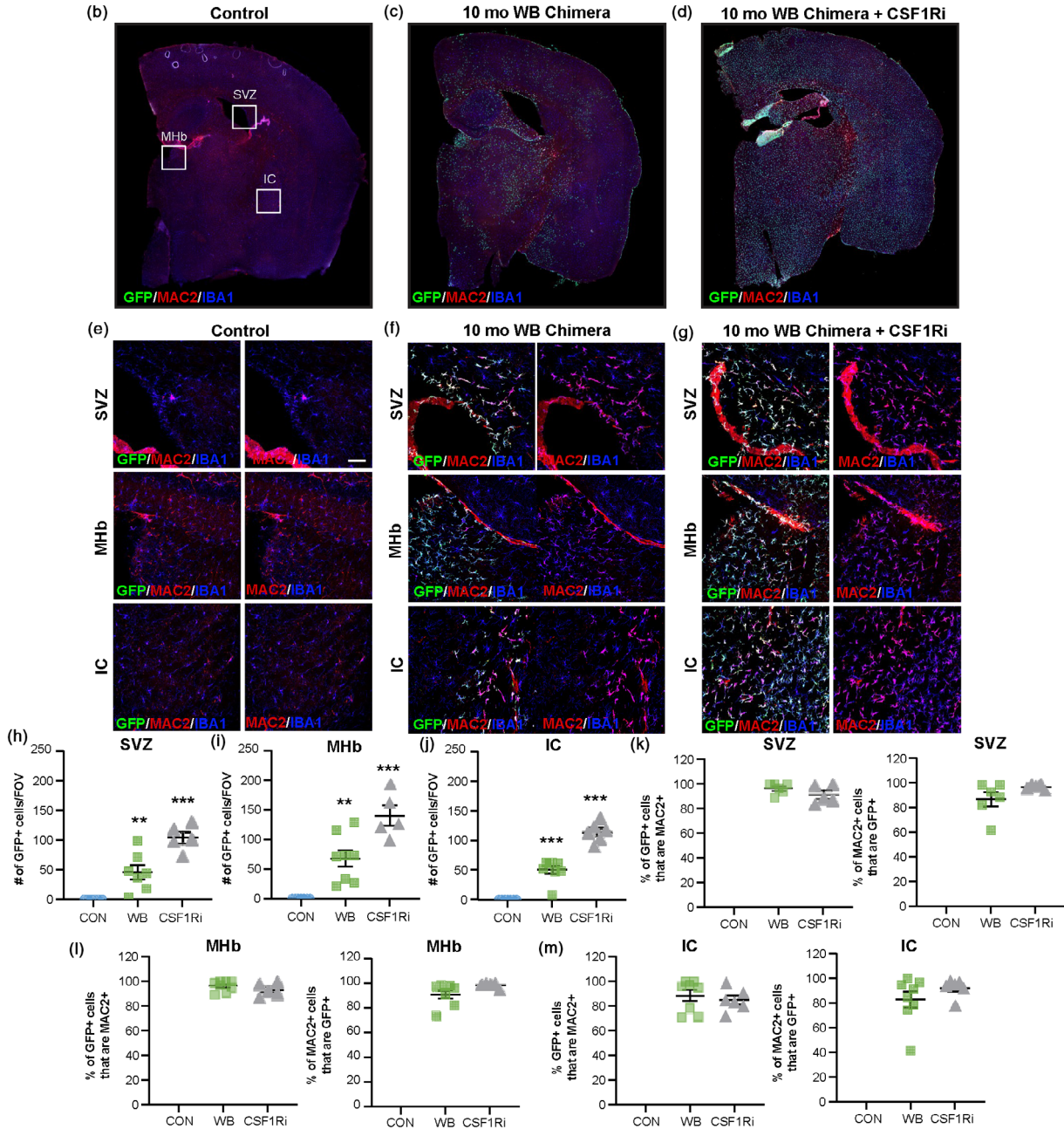


FIGURE 2 Legend on next page.

CCR2 and CD14 are downregulated following monocyte differentiation into macrophages (Fantuzzi et al., 1999; Steinbach & Thiele, 1994; Wong et al., 1997) and studies using CCR2<sup>RFP</sup> knock-in mice report that CCR2<sup>RFP</sup> cells are not detected in the CNS, even under inflammatory conditions (Saederup et al., 2010), meaning that these markers fail to label monocyte-derived cells past a few days following engraftment in the CNS. Given these difficulties, we next sought to examine whether MAC2 could serve as (1) a marker for BM-derived cells and (2) a long-lasting marker for these cells once engrafted in the brain.

To accomplish this, we utilized BM GFP chimeric mice to assess the ability of this monocyte/macrophage marker candidate to label infiltrating BM-derived cells. CAG-GFP donor BM was administered (via retro-orbital injection) to whole body (WB, with >95% chimerism) irradiated mice and allowed to recover for ~10 months, resulting in long-term GFP chimeric mice. In this model, GFP+ expression indicates that cells derive from the BM. Following recovery, we explored co-localization of MAC2 expression in GFP+ cells in the adult mouse brains of long-term GFP BM chimeric mice and long-term GFP BM chimeric mice that underwent colony-stimulating factor 1 receptor inhibitor (CSF1Ri)-induced myeloid cell depletion and repopulation (~6 months following CSF1Ri treatment and CNS engraftment) (Figure 2a). In a previous study, we demonstrated that irradiation/BM transplant followed by CSF1Ri treatment results in substantial replacement of the microglial compartment with BM-derived monocytes (Hohsfield et al., 2020), providing a model to evaluate long-term and brain-wide BM-derived monocyte/macrophage engraftment.

Ten-month WB irradiated mice exhibited ~20% infiltration of GFP+ cells, with prominent deposition observed in areas near the lateral ventricle/subventricular zone (SVZ), medial habenula (MHb), internal capsule (IC), and meninges (Figure 2a and b). In accordance with our prior data, we observed that these infiltrating GFP+ cells express little to no levels of P2RY12 and TMEM119, two canonical microglial markers (data not shown). Ten-month WB irradiated mice treated with CSF1Ri exhibited 79–96% (mean = 86%) engraftment of GFP+ cells, seen throughout the brain parenchyma (Figure 2a, d, and h–j). In aged-matched controls, no GFP+ cell deposition is visible (Figure 2b, and h–j). Notably, we found that MAC2+ staining was still apparent in GFP+/IBA1+ cells, even 6 and 10 months after CNS infiltration was stimulated, while no MAC2

expression was seen in control animals or in GFP-/IBA1+ cells (Figure 2b–g, and h–j). In 10 mo WB irradiated mice, an average of 89–96% of GFP+ cells express MAC2 near the lateral ventricle/SVZ, MHb, and IC (Figure 2k–m). In 10 mo WB irradiated + CSF1Ri treated mice, an average of 85–93% of GFP+ cells express of MAC2 near the lateral ventricle/SVZ, MHb, and IC (Figure 2k–m). It should be noted that there are differential levels of MAC2+ staining in GFP+ cells in mice, which likely reflects the more recent infiltration of BM-derived cells into the parenchyma, indicating that MAC2 expression may gradually decrease over time as cells become more CNS parenchymal macrophage- or microglia-like to fill the tissue niche. Some MAC2 expression is seen in the nuclei of resident microglia, but clearly fills the cell body and processes of GFP +/IBA+ cells. Given the high level of co-expression of MAC2 in GFP BM-derived myeloid cells, these results indicate that MAC2 can serve as a histological marker of BM-derived infiltrating cells and utilized as a potential long-lasting marker to distinguish between peripherally derived monocytes/macrophages and resident microglia.

### 3.3 | MAC2 is not a ubiquitous marker for plaque-associated microglia during the early stages of disease in 5xFAD mice

While we identified MAC2 as a marker for peripheral infiltrates, *Lgals3* has also been reported in specific myeloid cell populations under certain conditions. For example, *Lgals3* was identified as an upregulated gene in amyloid- $\beta$  (A $\beta$ ) plaque-associated microglia, also known as disease-associated microglia (DAM) or microglial neurodegenerative (MGnD) phenotype (Butovsky & Weiner, 2018; Krasemann et al., 2017), and in isolated amyloid plaque-containing microglia (Grubman et al., 2021). Moreover, galectin-3/MAC2 staining has been found in microglia in close contact with A $\beta$  plaques in both human and mice (Boza-Serrano et al., 2019). However, it is often difficult to distinguish between monocytes/macrophages and activated microglia, as they share many morphological and gene expression similarities (Bennett et al., 2016; Butovsky et al., 2014; Buttgerit et al., 2016; Keren-Shaul et al., 2017; Konishi et al., 2017). To directly test *Lgals3*/Galectin-3 expression in the AD brain, we stained for MAC2 in 5xFAD mice, and then used JHMV infection to trigger peripheral infiltration,

**FIGURE 2** MAC2 is a specific and long-lasting marker for bone marrow-derived infiltrating cells. (a) Schematic of experimental design for the generation of long-term green fluorescent protein (GFP) bone marrow (BM) chimeras. Treatment of BM chimeras with colony-stimulating factor 1 receptor (CSF1R) inhibitor results in extensive parenchymal engraftment of BM-derived peripheral myeloid cells in the brain (Hohsfield et al., 2020). Four-month-old wild type (WT) mice underwent whole body (WB) irradiation (1000 cGy) and BM transplant via retro-orbital injection of  $2 \times 10^6$  BM cells from age- and sex- matched CAG-GFP mice. Following a 3.5 mo recovery, mice were treated with CSF1R inhibitor PLX3397 (600 ppm) for 14d, and then placed on control diet for 6 mo. (b–g) Representative images of whole coronal brain sections (b–d) and high resolution 20 $\times$  confocal (e–g) images of GFP+ (green), MAC2+ (red) and IBA1+ (red) cells in control (b, e), ~10 mo WB chimeras (c, f), and ~10 mo WB chimeric mice treated with a CSF1R inhibitor (d, g). White boxes in (b) indicate brain regions of interest shown in (e–g). Images show that MAC2+ cells co-localize with GFP BM-derived cells in the brain parenchyma. (h–j) Quantification of the number of GFP+ cells per field of view (FOV) in the subventricular zone (SVZ) (h), medial habenula (MHb) (i), and internal capsule (IC) (j) in control, ~10 mo WB chimeras, and ~10 mo WB chimeric mice treated with a CSF1R inhibitor. (k–m) Percentage of GFP+ cells that express MAC or MAC2+ cells that express GFP in the subventricular zone (SVZ) (k), medial habenula (MHb) (l), and internal capsule (IC) (m) in control, ~10 mo WB chimeras, and ~10 mo WB chimeric mice treated with a CSF1R inhibitor. Data are represented as mean  $\pm$  SEM ( $n = 5-9$ ). \*\*  $p < .01$ , \*\*\*  $p < .001$ , compared to control. Scale bar ~60  $\mu$ m



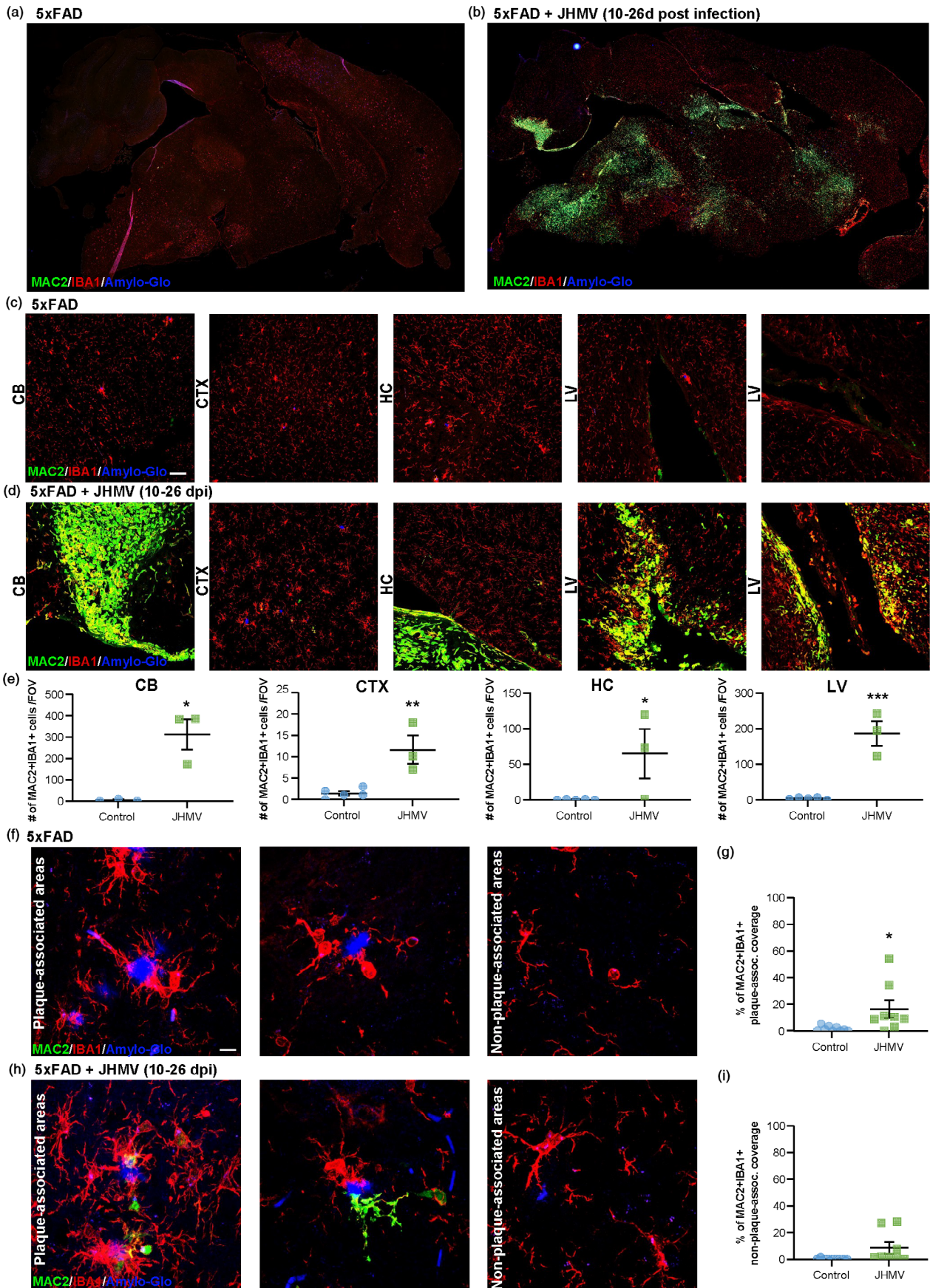


FIGURE 3 Legend on next page.

evaluating whether MAC2 expression is specific to plaque-associated microglia.

5xFAD mice, which express five human APP and PSEN1 transgenes with familial Alzheimer's disease mutations, display extensive extracellular plaque deposition and microgliosis (Oakley et al., 2006). In these mice, amyloid plaques begin to form around 3 months of age and exhibit significant plaque deposition and microglial activation in the cerebral cortex and brain stem, including the thalamus, midbrain, and hindbrain, by 4 months of age (Figure 3a). Here, we i.c. infected 4-month-old 5xFAD mice with JHMV to stimulate monocyte infiltration into the CNS. Brain sections of uninfected and JHMV-infected mice were stained for amyloid plaques (Amylo-Glo), myeloid cells (IBA1), and MAC2. Evaluation of whole brain sagittal sections showed profound MAC2 staining in JHMV-infected 5xFAD (Figure 3b), but not uninfected mice (Figure 3a). Similar to WT JHMV-infected mice, MAC2<sup>+</sup> cell deposition is apparent in areas near ventricles (in the cerebellum near the fourth ventricle, in tissue near the lateral ventricle) and white matter tracts (the corpus callosum, middle longitudinal fasciculus), whereas little to no MAC2 staining is present in 5xFAD controls (Figure 3a–e). MAC2<sup>+</sup> cells are seen in the choroid plexus of 5xFAD controls, but do not infiltrate into the parenchyma (Figure 3c and e). High resolution images show little to no MAC2<sup>+</sup> staining in plaque-associated microglia (1.6% of MAC2 staining in plaque-associated IBA1<sup>+</sup> cells) in uninfected 5xFAD mice (Figure 3f–g), and an average of 16.4% of MAC2 staining in IBA1<sup>+</sup> cells surrounding amyloid plaques in JHMV-infected 5xFAD mice (Figure 3h–i). It also appears that not all plaque-associated microglia are MAC2<sup>+</sup> nor is there an abundance of MAC2<sup>+</sup> IBA1<sup>+</sup> in plaque-associated regions. MAC2<sup>+</sup> staining is also present in non-plaque-associated regions in IBA1<sup>+</sup> cells (8.7% MAC2<sup>+</sup> IBA1<sup>+</sup> staining) in JHMV-infected mice (Figure 3d, and h–i). These findings provide evidence that MAC2 is not a specific marker for plaque-associated microglia and is not expressed by microglia in the young non-infected 5xFAD mouse model, indicating that *Lgals3*/MAC2 expression may reflect peripheral infiltrates honing to plaques.

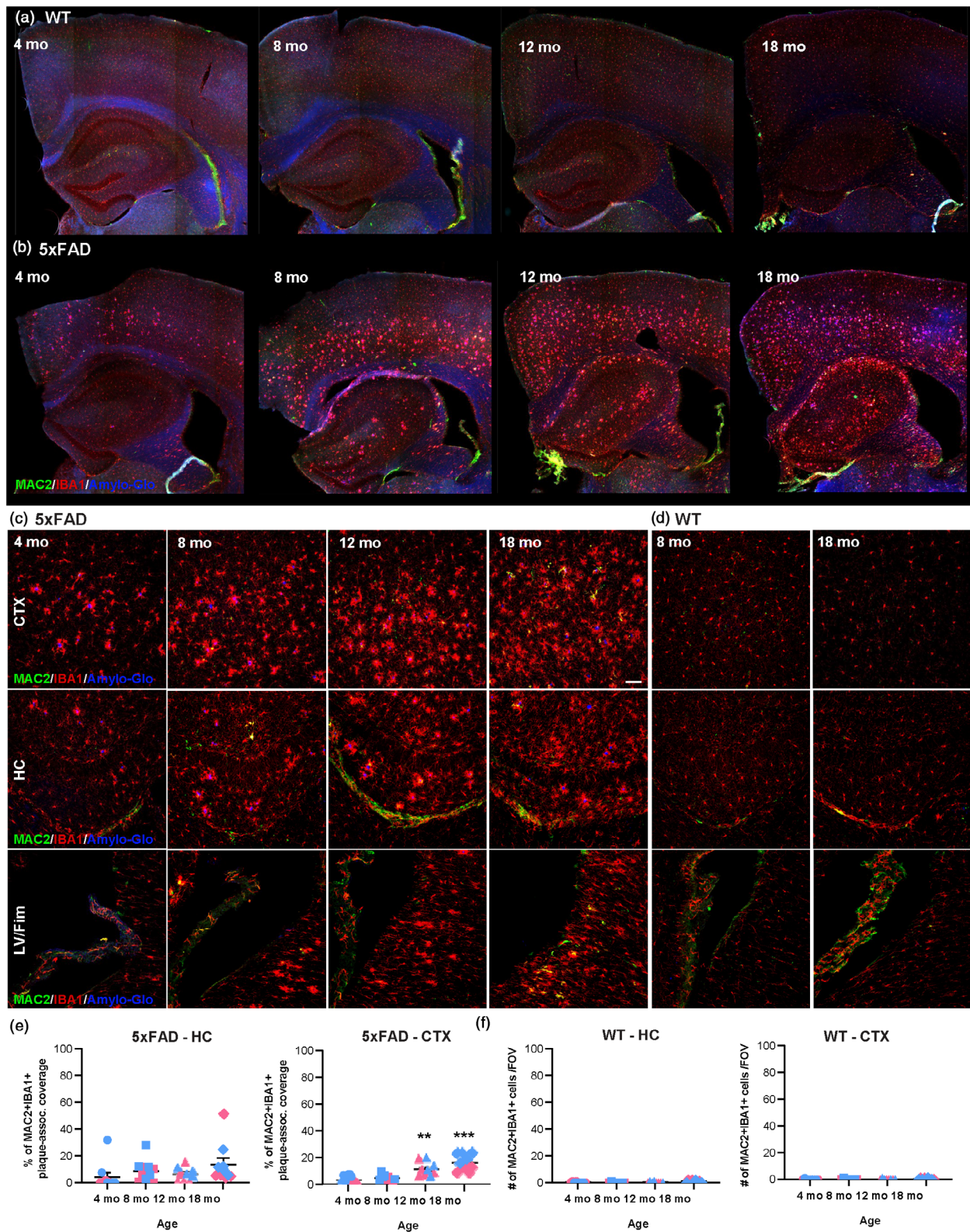
### 3.4 | The effects of aging on MAC2 staining in WT and 5xFAD mice

Transcriptional profile analysis has revealed an upregulation of *Lgals3* in microglia isolated from mouse models of neurodegenerative

disease, including Alzheimer's disease and amyotrophic lateral sclerosis as well as aging, and identified *Lgals3* as a hub gene for primed microglia (Holtman et al., 2015). Safaiyan et al. recently identified white matter-associated microglia (WAM), which accumulate in white matter tracts during aging. *Lgals3* was one of 39 transcripts of the WAM signature that was identified when comparing aged white matter compared to young white matter or aged gray matter myeloid cells; and the authors report expression of galectin-3 in IBA1<sup>+</sup> cells in old but not young white matter (Safaiyan et al., 2021).

To address whether MAC2 staining also increases with aging under steady-state and diseased conditions, we next stained brain sections from 4, 8, 12, and 18 mo WT and 5xFAD mice with MAC2, IBA1, and Amylo-Glo. Again, we observe that MAC2<sup>+</sup> IBA1<sup>+</sup> cells and staining is most apparent in the choroid plexus of WT and 5xFAD mice, regardless of age (Figure 4a and b). In line with previous studies, our data shows an age-dependent increase in MAC2<sup>+</sup> IBA1<sup>+</sup> cell accumulation in 5xFAD mice in the hippocampus, cortex, and in white matter areas (e.g. fimbria, corpus callosum) within close proximity to the lateral ventricle (Figure 4b, c, and e). In gray matter regions, the majority of MAC2<sup>+</sup> cells are IBA1<sup>+</sup> plaque-associated microglia (Figure 4b and c). However, there appears to be a lag in the appearance of MAC2 expression and microglial/DAM activation around plaques. For example, prominent MAC2 expression in plaque-associated IBA1<sup>+</sup> cells appears at 18 mo of age in the cortex despite the accumulation of these plaque-associated microglia at 4 mo of age (Figure 4c). Furthermore, the majority of IBA1<sup>+</sup> plaque-associated cells do not express MAC2 (<20% of IBA1<sup>+</sup> cells are MAC2<sup>+</sup>; Figure 4e), meaning MAC2 does not account for a significant proportion of plaque-associated cells. MAC2<sup>+</sup> IBA1<sup>+</sup> cell deposition does not occur in gray matter areas in the aged WT mice (Figure 4a, d, and f); however, interestingly we do observe MAC2<sup>+</sup> IBA1<sup>+</sup> cells present in white matter areas at 18 mo of age (Figure 4d). This is also true for 5xFAD mice (Figure 4c). Thus, it appears that MAC2 expression is not a specific response to plaques. Given the proximity of these brain regions to the lateral ventricles and the lack of MAC2 expression in the majority of plaque-associated microglia, we postulate two scenarios: (1) a specific subpopulation of activated microglia (subset of DAM and/or WAM) switch on *Lgals3* and galectin-3/MAC2 expression over time or (2) that MAC2<sup>+</sup> cells derive from a different cell source, such as from the periphery. Since microglia and macrophages undergo rapid morphological and transcriptional transformations upon activation, reflective of the highly plastic nature of myeloid cells, defining MAC2

**FIGURE 3** MAC2 is not a ubiquitous marker for plaque-associated microglia during the early stages of disease in 5xFAD mice. Four-month-old 5xFAD mice were infected intracranially with 200 PFU of JHMV strain and brains evaluated at 10–25 d.p.i. (a–d) Representative whole sagittal brain section (a–b) and 20× confocal (c–d) images of Amylo-Glo (amyloid, blue), MAC2 (green), and IBA1 (red) staining in control (a, c) and JHMV-infected (b, d) 5xFAD mice. (e) Quantification of the number of MAC2<sup>+</sup> IBA1<sup>+</sup> cells per field of view (FOV) in the cerebellum (CB), cortex (CTX), hippocampus (HC), and lateral ventricle (LV). (f, h) Representative high resolution 100× confocal images of Amylo-Glo + plaques (amyloid, blue), MAC2<sup>+</sup> (green) cells, and IBA1<sup>+</sup> (red) cells in plaque-associated and non-plaque-associated areas in the brains of control (f) and JHMV-infected (h) 5xFAD mice. Images show that MAC2<sup>+</sup> cells do not label plaque-associated microglia in non-infected 5xFAD mice. (g,i) Quantification of % MAC2<sup>+</sup> IBA1<sup>+</sup> staining in IBA1<sup>+</sup> cells and in plaque-associated (g) and non-plaque-associated (i) areas of the cortex and hippocampus. Multiple field of views in these brain regions were quantified across three animals from each group. Data are represented as mean ± SEM (n = 8–9). \* p < .05. Scale bar in (c) ~60 μm; in (e) ~10 μm



**FIGURE 4** The effects of aging on MAC2 staining in WT and 5xFAD mice. Brains of wild type (WT) and 5xFAD mice at 4, 8, 12, and 18 months (mo) of age were evaluated for MAC2 staining in plaque-associated microglia. (a–b) Representative images of MAC2 (green), IBA1 (red, myeloid cells), and Amylo-Glo (blue, amyloid plaques) staining in brain sections of WT (a) and 5xFAD (b) mice. (c–d) Representative 20× confocal images of MAC2, IBA1, and Amylo-Glo staining in the cortex (CTX), hippocampus (HC), and lateral ventricle (LV)/fimbria (Fim) of WT (d) and 5xFAD (c) mice shows the age-dependent increase in MAC2 staining in 5xFAD mice. (e–f) Quantification of plaque-associated MAC2+ IBA1+ staining and MAC2+ IBA1+ cells in 5xFAD and WT mice, respectively. Female (pink) and male (blue) mice are indicated by color scheme. Data are represented as mean ± SEM (n = 3–5). \*\* p < .01, \*\*\* p < .001, compared to 4 mo. Scale bar in (c) ~60 μm

expression as an indicator of cell activation or peripheral infiltration remains difficult. However, given the findings in this study, specifically the spatial and temporal expression of MAC2<sup>+</sup> IBA1<sup>+</sup> cells in areas that are closely associated with peripheral immune cell infiltration in CNS disorders, most prominently in white matter areas in multiple sclerosis, we hypothesize that MAC2 expression labels a subset of peripheral immune cells in the young adult brain that can infiltrate and engraft the brain.

## 4 | DISCUSSION

Microglia and BM-derived monocytes are implicated in a number of neurological disorders (Herz et al., 2017). In AD, several genome-wide association studies have identified variants associated with myeloid cells that increase disease risk, highlighting the importance of myeloid cells and the growing need to decipher their phenotype and function during disease (Bradshaw et al., 2013; Guerreiro et al., 2013; Jonsson et al., 2013; Lambert et al., 2013). However, myeloid cells are highly dynamic cells, which can be influenced at both the transcriptional and protein level by specific diseases during disease induction and progression, thus complicating the use of reliable and stable cell-specific markers and posing challenges to the functional exploration and contributions of these distinct cells. For instance, the presence of peripheral-derived myeloid cells around amyloid plaques and their contribution in AD pathology has been a long-standing question in the field with contrasting results (El Khoury et al., 2007; Hawkes & McLaurin, 2009; Jay et al., 2015; Reed-Geaghan et al., 2020; Simard et al., 2006).

Under steady state conditions, microglia and monocyte/macrophages populations express several overlapping surface markers, including Cd11b, F4/80, CD115 (CSF1R), and IBA1 (Gautier et al., 2012; Prinz et al., 2011). Recent gene expression studies have identified surface markers and transcription factors unique to resident microglia (e.g., P2RY12, TMEM119, SIGLEC-H, and *Sall1*), but several of these markers lose their distinct homeostatic expression levels during disease/cell activation (Bennett et al., 2016; Butovsky et al., 2014; Buttgerit et al., 2016; Chen & Colonna, 2021; Keren-Shaul et al., 2017; Konishi et al., 2017). Despite the utility of previous approaches (e.g., BM chimeras, parabiosis, and mouse models of fluorescent protein expression or tamoxifen-inducible Cre-recombinase under monocyte/microglia promoters) in providing myeloid cell-specific labeling/targeting and unprecedented opportunities to study microglia and monocytes (Ajami et al., 2007; Jung et al., 2000; Mildner et al., 2007; Wang et al., 2016), the field has lacked a commercially available histological marker for monocyte-derived populations in the CNS.

In this study, our main goal was to identify a marker that can discern between murine microglial and monocyte-derived cell populations during inflammation and disease in the CNS. Given the plasticity of myeloid cells, we recognize that the identification of cell lineage based on a single marker is not ideal (Bennett et al., 2016; Butovsky et al., 2014); however, previous studies have identified unique single genes and markers for microglia, such as P2ry12 and *Tmem119*, that have aided investigations and added to the repertoire

of tools utilized by CNS myeloid cell researchers (Bennett et al., 2016; Butovsky et al., 2014). These markers have been indispensable to identifying endogenous and homeostatic microglia in the CNS. Here, we show that *Lgals3* is highly expressed in monocyte/macrophages in the healthy and JHMV-infected brain, and that galectin-3/MAC2 can be used as a histological marker to distinguish peripherally derived myeloid cells from microglia under both steady state and neuro-inflammatory conditions. Galectin-3/MAC2 is a  $\beta$ -galactoside-binding lectin that is highly expressed in and secreted by monocytes/macrophages; it is associated with many macrophage functions, including cell adhesion, migration, proliferation, and apoptosis (MacKinnon et al., 2008). However, galectin-3 expression has also been reported in other cell populations, including fibroblasts, osteoblasts, endothelial cells, neuronal cells, and immune cells (neutrophils, eosinophils, basophils, mast cells) (Thomas & Pasquini, 2018). Here, we show that *Lgals3* is not highly expressed in other Cd11b<sup>+</sup> cell populations via scRNAseq, including T cells, B cells, NK cells, neutrophils, and microglia, but is highly expressed in several monocyte and macrophage cell clusters. Although we do not observe significant MAC2<sup>+</sup> staining in non-IBA1<sup>+</sup> cells in the CNS, careful consideration here is also warranted and IBA1 (or other myeloid cell markers) should be employed in combination with MAC2 to distinguish these myeloid populations.

In line with our study, recent work by Ochocka et al. using scRNAseq and flow cytometry of CD11b<sup>+</sup> myeloid cells in naïve and glioma-bearing mice showed that *Lgals3* and galectin-3 are highly expressed by infiltrating monocyte/macrophages at RNA and protein levels, respectively (Ochocka et al., 2021). These authors propose the use of TMEM119 and galectin-3 for efficient and distinguishable separation of microglia and monocyte/macrophages in glioma models and highlighted their ability to achieve distinct spatial distribution of these cells within tumor tissue (Ochocka et al., 2021). Moreover, recent studies in mouse models of diabetes and spinal cord injury have identified unique populations of infiltrating and phagocytic macrophages, distinct from resident microglia, that express *Lgals3*/galectin-3/MAC2. In diabetes, galectin-3 expressing macrophages infiltrate and aggregate near injury sites, implicating these cells in the response to cerebrovascular insults (Mehina et al., 2021). In spinal cord injury, injury-activated macrophages maintain a transcriptionally distinct identity to activated microglia, expressing higher levels of *Lgals3*, *ApoE*, and *Cxcr4*, among other genes (Wahane et al., 2021). In the present study, we also observed distinct spatial distribution of MAC2<sup>+</sup> cells in the JHMV-infected brain; cells were sequestered in the brain stem, white matter areas, and regions surrounding the ventricles. Further study is needed to evaluate the distinct contributions of MAC2<sup>+</sup> myeloid cells and MAC2<sup>-</sup> myeloid cells in pathogenesis, but given the role of BM-derived myeloid cells in demyelination (Yamasaki et al., 2014), it is not surprising to find their selective recruitment in these areas. In GFP BM chimeras, MAC2<sup>+</sup> cells were observed in the choroid plexus and perivascular spaces in whole body-irradiated mice, respectively, consistent with their partial cell turnover from BM sources (Goldmann et al., 2016; Van Hove et al., 2019). Identifying MAC2 as a distinct histological marker for monocytes provides investigators with the ability

to explore the spatial distribution and dynamics of these peripheral cells without the side effects of irradiation or other experimental caveats (e.g., tamoxifen).

In mice, monocytes express CD11b and CD45, and are divided into two main subpopulations: classical/inflammatory monocytes (Ly6C<sup>hi</sup>CCR2 + CX3CR1<sup>lo</sup>) and non-classical/patrolling monocytes (Ly6C<sup>lo</sup>CCR2<sup>lo</sup> CX3CR1<sup>hi</sup>) (Geissmann et al., 2003; Geissmann et al., 2010). Of interest, studies have shown that *Lgals3* is enriched in a population of Ly6C<sup>+</sup> monocytes located in the BM and blood (Mildner et al., 2017). Although transcriptional profile analyses of microglia and monocytes have generated several proposed monocyte-specific markers based off of an identified monocyte signature, including *ApoE*, *Ccr2*, *Ms4a7*, and *Clec12a* (Bennett et al., 2018; Cronk et al., 2018; Hohsfield et al., 2020; Lund et al., 2018; Anat Shemer et al., 2018) these markers have presented a number of limitations: upregulation during disease, downregulation upon macrophage differentiation, or lack of success in staining fixed adult brain tissue. For example, though *Cd11a* (*Itgal*) is expressed on all peripheral circulating immune cells and not microglia, it could only be detected on infiltrating cells in fixed brain sections using a tedious tyramide amplification strategy, limiting its practical use (Shukla et al., 2019). In addition, Bennett et al. identified MS4A7 as a unique marker for HSC-derived cells, but MS4A7 staining was only shown in blood, fetal brain, and primary human microglia, and RNA in situ hybridization was required to show staining in postmortem AD brain samples (Bennett et al., 2018). CD44 was a marker identified for distinguishing CNS infiltrating from CNS resident cells by Korin et al., however, later found by Mrdjen et al. to be upregulated in microglia during disease or aging (Korin et al., 2017; Mrdjen et al., 2018). Given that monocytes and macrophages exist as heterogenous populations with varying expression profiles (reflecting functional diversity, differential origins, differentiation status, tissue of residence) and dynamic adaptation of their transcription regulatory networks to changing environments (Gosselin et al., 2014; Lavin et al., 2014), it has been challenging for researchers to develop a marker for monocyte-derived cells that remains intact following differentiation and tissue engraftment. Here, we show that MAC2 staining is retained in BM-derived cells 6–10 months following CNS recruitment in the brain, indicating that MAC2 may be a marker that is conserved across several monocyte and macrophages subpopulations, or at least the subsets recruited to the CNS/sites of inflammation.

Transcriptional profile analysis of isolated microglia from mouse models of AD, amyotrophic lateral sclerosis, and aging identified *Lgals3* as a central hub gene in primed microglia, along with *ApoE*, *Axl*, *Clec7a*, and *Itgax* (Holtman et al., 2015). Further comprehensive scRNAseq analysis of microglia in neurodegenerative diseases discovered a unique microglial subset, termed disease-associated microglia (DAM) or microglial neurodegenerative phenotype (MGnD) microglia. These cells exhibit a downregulation in homeostatic microglia genes (e.g., *P2ry12* and *Tmem119*) and upregulation in genes involved in phagocytic, lysosomal, and lipid metabolism pathways (e.g., *ApoE*, *Axl*, *Clec7a*, *Cst7*, *Itgax*, and *Lilrb4*) (Keren-Shaul et al., 2017), including *Lgals3* (Butovsky & Weiner, 2018; Krasemann et al., 2017), which is

upregulated during later stages of neurodegeneration (Chen & Colonna, 2021). However, a recent study has shown that enzymatic dissociation methods can induce aberrant gene expression signatures in microglia (Marsh et al., 2020), indicating that some scRNAseq findings may be confounded and an artifact of isolation methods, analysis, or sequencing platform. A recent investigation utilizing scRNAseq, snRNAseq and scATACseq identified distinct microglia subpopulations in 3xTgAD and 5xFAD mice, including a DAM cluster containing *Cst7*, but lacking *Lgals3*; instead, *Lgals3* was found in a small cluster containing *Cd14* and *Itgal* (Balderrama-Gutierrez et al., 2021). Galectin-3 was also shown to be significantly elevated in human AD patients, exhibit preferential expression in microglia in contact with plaques, and serve as a ligand for TREM2 (Boza-Serrano et al., 2019). In contrast, Sobue et al. showed that gene expression levels of *LGALS3*, *AIF1*, and *CD68*, were unchanged in human AD precuneus samples (Sobue et al., 2021). In line with this, using bulk-tissue RNAseq analysis, our lab has shown that while *Lgals3* is upregulated in 5xFAD mice in the hippocampus and thalamus compared to wild types, expression levels do not change following sustained microglial depletion, unlike several homeostatic (e.g., *Csf1r*, *Cx4cr1*, *Hexb*, and *Siglech*) and DAM genes (e.g., *Clec7a*, *Cst7*, and *Itgax*) which are significantly downregulated by microglial depletion (Spangenberg et al., 2019). These data provide evidence that elevated *Lgals3* in 5xFAD brains may not be specific to microglia. Consistent with spatial transcriptomics and in situ sequencing data in APP<sup>NL-G-F</sup> mice, *Lgals3* was not identified in plaque-induced genes (PIGs), consisting of 57 genes that overlap with several DAM genes (Chen, Lu, et al., 2020). The current study shows that MAC2 staining is not present in plaque-associated microglia in the young 5xFAD mice, and only appears in plaque-associated IBA1<sup>+</sup> cells during JHMV infection or in aged animals (albeit at modest numbers). A recent study—showing clonal hematopoiesis of indeterminate potential (CHIP) is associated with protection against AD—detected mutated marrow-derived cells in the brains of patients which appear indistinguishable from microglia (Bouzid et al., 2021), highlighting the potential role for peripheral infiltrates in replacing defective microglia during aging or disease. Thus, the presence of MAC2<sup>+</sup> cells in disease and experimental models should be considered a potential sign of peripheral infiltrates, and subsequently followed up with BM chimeric models, parabiosis, or BM lineage tracing transgenics. It is possible that microglia may upregulate *Lgals3* under certain conditions, including infection or inflammatory stimulus, or that upregulated mRNA does not effectively translate to protein expression. Likewise, we cannot discount the possibility that microglia in other conditions (stroke, injury, and chronic neurodegenerative disease) may also upregulate *Lgals3*, or that these MAC2<sup>+</sup> cells may derive from BM sources. Other explanations for the disparity between *Lgals3* transcript levels and MAC2 staining in plaque-associated microglia could be a result of: (1) microglia and infiltrating peripheral myeloid cells expressing different isoforms of *Lgals3*/galectin-3, in which one isoform is detectable by the MAC2 antibody while the other is not, or (2) different secretion and/or cellular retention rates of *Lgals3*/galectin-3 within these two myeloid cell populations. Moreover, *Lgals3* may also be expressed by discrete microglial populations in the brain—for example, in specialized

axon tract-associated microglia (ATM) or P7/early postnatal microglia (termed proliferative region-associated microglia (PAM), which appear as amoeboid cells that transiently reside in axon tracts of the corpus callosum and cerebellum (Hammond et al., 2019; Li et al., 2019), as well as in white matter-associated microglia (WAM), found specifically in white matter areas that increase with aging (Safaiyan et al., 2021). Furthermore, surviving microglia following CSF1Ri-induced depletion have been described as MAC2+ (Zhan et al., 2020). Given these findings and the data from this current study, we would implore researchers to consider *Lgals3*/MAC2 expression as a potential indicator of peripheral origins, but encourage further validation with other important tools, such as fate-mapping techniques. For example, lineage tracing from *Ms4a3* or *Cxcr4* could be utilized, as it has recently been shown as a specific gene expressed by BM-derived granulocyte-monocyte progenitors (GMPs) or HSC-derived monocytes, respectively, to efficiently trace monocyte-derived cells (Liu et al., 2019; Werner et al., 2020). Other markers include Cd49d, also expressed by T cells and DCs, and Cd49e, which were identified in peripheral monocyte populations, but not in CNS resident myeloid cell populations utilizing CyTOF (Bahareh Ajami et al., 2018).

In sum, we identify galectin-3/MAC2 as a potential monocyte marker that can be utilized in inflamed CNS tissue. Unlike other markers, BM-derived myeloid cells retain MAC2 expression even as they leave perivascular areas and occupy the parenchymal niche for prolonged periods of time. The ability to discern peripherally derived cells from resident microglia is critical to exploring their distinct roles in health and disease. Growing attention has been placed on understanding CNS myeloid cells and the environmental cues that sculpt their transcriptional and phenotypic profiles, particularly during disease. Detailed knowledge of these distinct myeloid cell populations and our ability to distinguish microglia and monocytes will prove essential in targeting these cells for improved therapeutic strategies.

## ACKNOWLEDGMENTS

We thank Edna Hingco for her excellent technical assistance. WT and 5xFAD time course tissue was provided by U54 AG054349 (NIA Model Organism Development and Evaluation for Late onset Alzheimer's Disease [MODEL-AD]). K.N.G. was supported by the National Institutes of Health (NIH) under awards: R01NS083801 (NINDS), RF1AG056768 (NIA), and RF1AG065329 (NIA). L.A.H. was supported by the Alzheimer's Association Research Fellowship (AARF-16-442762). T.E.L. was supported by the National Institutes of Health (NIH) under awards: R01NS041249 (NINDS) and R35NS116835 (NINDS), the National Multiple Sclerosis Society (NMSS) Collaborative Research Center grant CA-1607-25040 and The Ray and Tye Noorda Foundation. Y.G. was supported by NIH T32 training grant (NS082174).

## CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

## AUTHOR CONTRIBUTIONS

L.A.H. developed experimental protocols, designed, performed, and analyzed experiments and wrote the manuscript. K.I.T., Y.G., A.R.S.,

S.J.K., Y.C. and S.F. performed, and analyzed experiments. M.A.I. and T.E.L. contributed to project design. K.N.G. directed the project, designed the experiments, interpreted the results and wrote the manuscript.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from TEL and KNG upon reasonable request.

## ORCID

Matthew A. Inlay  <https://orcid.org/0000-0002-0451-2076>

Thomas E. Lane  <https://orcid.org/0000-0003-0392-0825>

Kim N. Green  <https://orcid.org/0000-0002-6049-6744>

## REFERENCES

- Ajami, B., Bennett, J. L., Krieger, C., McNagny, K. M., & Rossi, F. M. (2011). Infiltrating monocytes trigger EAE progression, but do not contribute to the resident microglia pool. *Nature Neuroscience*, *14*(9), 1142–1149. <https://doi.org/10.1038/nn.2887>
- Ajami, B., Bennett, J. L., Krieger, C., Tetzlaff, W., & Rossi, F. M. (2007). Local self-renewal can sustain CNS microglia maintenance and function throughout adult life. *Nature Neuroscience*, *10*(12), 1538–1543. <https://doi.org/10.1038/nn2014>
- Ajami, B., Samusik, N., Wieghofer, P., Ho, P. P., Crotti, A., Bjornson, Z., Prinz, M., Fantl, W. J., Nolan, G. P., & Steinman, L. (2018). Single-cell mass cytometry reveals distinct populations of brain myeloid cells in mouse neuroinflammation and neurodegeneration models. *Nature Neuroscience*, *21*(4), 541–551. <https://doi.org/10.1038/s41593-018-0100-x>
- Balderrama-Gutierrez, G., Liang, H., Rezaie, N., Carvalho, K., Forner, S., Matheos, D., Rebboah, E., Green, K. N., Tenner, A. J., LaFerla, F., & Mortazavi, A. (2021). Single-cell and nucleus RNA-seq in a mouse model of AD reveal activation of distinct glial subpopulations in the presence of plaques and tangles. *bioRxiv*. <https://doi.org/10.1101/2021.09.29.462436>
- Bennett, F. C., Bennett, M. L., Yaqoob, F., Mulinyawe, S. B., Grant, G. A., Hayden Gephart, M., Plowey, E. D., & Barres, B. A. (2018). A combination of ontogeny and CNS environment establishes microglial identity. *Neuron*, *98*(6), 1170–1183. <https://doi.org/10.1016/j.neuron.2018.05.014>
- Bennett, M. L., Bennett, F. C., Liddel, S. A., Ajami, B., Zamanian, J. L., Fernhoff, N. B., Mulinyawe, S. B., Bohlen, C. J., Adil, A., Tucker, A., Weissman, I. L., Chang, E. F., Li, G., Grant, G. A., Hayden Gephart, M. G., & Barres, B. A. (2016). New tools for studying microglia in the mouse and human CNS. *Proceedings of the National Academy of Sciences of the United States of America*, *113*(12), E1738–E1746. <https://doi.org/10.1073/pnas.1525528113>
- Bergmann, C. C., Lane, T. E., & Stohlman, S. A. (2006). Coronavirus infection of the central nervous system: Host-virus stand-off. *Nature Reviews. Microbiology*, *4*(2), 121–132. <https://doi.org/10.1038/nrmicro1343>
- Blanc, C. A., Grist, J. J., Rosen, H., Sears-Kraxberger, I., Steward, O., & Lane, T. E. (2015). Sphingosine-1-phosphate receptor antagonism enhances proliferation and migration of engrafted neural progenitor cells in a model of viral-induced demyelination. *The American Journal of Pathology*, *185*(10), 2819–2832. <https://doi.org/10.1016/j.ajpath.2015.06.009>
- Bouzd, H., Belk, J. A., Jan, M., Qi, Y., Sarnowski, C., Wirth, S., Ma, L., Chrostek, M., Ahmad, H., Nachun, D., Yao, W., Beiser, A., Bick, A. G., Bis, J., Fornage, M., Longstreth, W. T., Lopez, O., Natarajan, P., & Jaiswal, S. (2021). Clonal hematopoiesis is associated with protection from Alzheimer's disease. *Blood*, *138*, 5. <https://doi.org/10.1101/2021.12.10.21267552>



- Boza-Serrano, A., Ruiz, R., Sanchez-Varo, R., García-Revilla, J., Yang, Y., Jimenez-Ferrer, I., Paulus, A., Wennström, M., Vilalta, A., Allendorf, D., Davila, J. C., Stegmayr, J., Jiménez, S., Roca-Ceballos, M. A., Navarro-Garrido, V., Swanberg, M., Hsieh, C. L., Real, L. M., Englund, E., ... Deierborg, T. (2019). Galectin-3, a novel endogenous TREM2 ligand, detrimentally regulates inflammatory response in Alzheimer's disease. *Acta Neuropathologica*, 138(2), 251–273. <https://doi.org/10.1007/s00401-019-02013-z>
- Bradshaw, E. M., Chibnik, L. B., Keenan, B. T., Ottoboni, L., Raj, T., Tang, A., Rosenkrantz, L. L., Imboywa, S., Lee, M., Von Korff, A., Alzheimer Disease Neuroimaging Initiative, Morris, M. C., Evans, D. A., Johnson, K., Sperling, R. A., Schneider, J. A., Bennett, D. A., & De Jager, P. L. (2013). CD33 Alzheimer's disease locus: Altered monocyte function and amyloid biology. *Nature Neuroscience*, 16(7), 848–850. <https://doi.org/10.1038/nn.3435>
- Butovsky, O., Jedrychowski, M. P., Moore, C. S., Cialic, R., Lanser, A. J., Gabrieli, G., Koeglsperger, T., Dake, B., Wu, P. M., Doykan, C. E., Fanek, Z., Liu, L., Chen, Z., Rothstein, J. D., Ransohoff, R. M., Gygi, S. P., Antel, J. P., & Weiner, H. L. (2014). Identification of a unique TGF-beta-dependent molecular and functional signature in microglia. *Nature Neuroscience*, 17(1), 131–143. <https://doi.org/10.1038/nn.3599>
- Butovsky, O., & Weiner, H. L. (2018). Microglial signatures and their role in health and disease. *Nature Reviews. Neuroscience*, 19(10), 622–635. <https://doi.org/10.1038/s41583-018-0057-5>
- Buttgereit, A., Lelios, I., Yu, X., Vrohings, M., Krakoski, N. R., Gautier, E. L., Nishinakamura, R., Becher, B., & Greter, M. (2016). Sall1 is a transcriptional regulator defining microglia identity and function. *Nature Immunology*, 17(12), 1397–1406. <https://doi.org/10.1038/ni.3585>
- Chen, H.-R., Sun, Y.-Y., Chen, C.-W., Kuo, Y.-M., Kuan, I. S., Tiger Li, Z.-R., Short-Miller, J. C., Smucker, M. R., & Kuan, C.-Y. (2020). Fate mapping via CCR2-CreER mice reveals monocyte-to-microglia transition in development and neonatal stroke. *Science Advances*, 6(35), eabb2119. <https://doi.org/10.1126/sciadv.abb2119>
- Chen, W. T., Lu, A., Craessaerts, K., Pavie, B., Sala Frigerio, C., Corthout, N., Qian, X., Laláková, J., Kühnemund, M., Voytyuk, I., Wolfs, L., Mancuso, R., Salta, E., Balusu, S., Snellinx, A., Munck, S., Jurek, A., Fernandez Navarro, J., Saido, T. C., ... de Strooper, B. (2020). Spatial transcriptomics and in situ sequencing to study Alzheimer's disease. *Cell*, 182(4), 976–991.e919. <https://doi.org/10.1016/j.cell.2020.06.038>
- Chen, Y., & Colonna, M. (2021). Microglia in Alzheimer's disease at single-cell level. Are there common patterns in humans and mice? *Journal of Experimental Medicine*, 218(9), e20202717. <https://doi.org/10.1084/jem.20202717>
- Chu, H. X., Arumugam, T. V., Gelderblom, M., Magnus, T., Drummond, G. R., & Sobey, C. G. (2014). Role of CCR2 in inflammatory conditions of the central nervous system. *Journal of Cerebral Blood Flow and Metabolism*, 34(9), 1425–1429. <https://doi.org/10.1038/jcbfm.2014.120>
- Cronk, J. C., Filiano, A. J., Louveau, A., Marin, I., Marsh, R., Ji, E., Goldman, D. H., Smirnov, I., Geraci, N., Acton, S., Overall, C. C., & Kipnis, J. (2018). Peripherally derived macrophages can engraft the brain independent of irradiation and maintain an identity distinct from microglia. *The Journal of Experimental Medicine*, 215(6), 1627–1647. <https://doi.org/10.1084/jem.20180247>
- Dickey, L. L., Worme, C. L., Glover, J. L., Lane, T. E., & O'Connell, R. M. (2016). MicroRNA-155 enhances T cell trafficking and antiviral effector function in a model of coronavirus-induced neurologic disease. *Journal of Neuroinflammation*, 13(1), 240. <https://doi.org/10.1186/s12974-016-0699-z>
- El Khoury, J., Toft, M., Hickman, S. E., Means, T. K., Terada, K., Geula, C., & Luster, A. D. (2007). Ccr2 deficiency impairs microglial accumulation and accelerates progression of Alzheimer-like disease. *Nature Medicine*, 13(4), 432–438. <https://doi.org/10.1038/nm1555>
- Elmore, M. R., Lee, R. J., West, B. L., & Green, K. N. (2015). Characterizing newly repopulated microglia in the adult mouse: Impacts on animal behavior, cell morphology, and neuroinflammation. *PLoS One*, 10(4), e0122912. <https://doi.org/10.1371/journal.pone.0122912>
- Elmore, M. R., Najafi, A. R., Koike, M. A., Dagher, N. N., Spangenberg, E. E., Rice, R. A., Kitazawa, M., Matusow, B., Nguyen, H., West, B. L., & Green, K. N. (2014). Colony-stimulating factor 1 receptor signaling is necessary for microglia viability, unmasking a microglia progenitor cell in the adult brain. *Neuron*, 82(2), 380–397. <https://doi.org/10.1016/j.neuron.2014.02.040>
- Fantuzzi, L., Borghi, P., Ciolli, V., Pavlakis, G., Belardelli, F., & Gessani, S. (1999). Loss of CCR2 expression and functional response to monocyte chemotactic protein (MCP-1) during the differentiation of human monocytes: Role of secreted MCP-1 in the regulation of the chemotactic response. *Blood*, 94(3), 875–883.
- Gautier, E. L., Shay, T., Miller, J., Greter, M., Jakubzick, C., Ivanov, S., Helft, J., Chow, A., Elpek, K. G., Gordonov, S., Mazloom, A. R., Ma'ayan, A., Chua, W. J., Hansen, T. H., Turley, S. J., Merad, M., & Randolph, G. J. (2012). Gene-expression profiles and transcriptional regulatory pathways that underlie the identity and diversity of mouse tissue macrophages. *Nature Immunology*, 13(11), 1118–1128. <https://doi.org/10.1038/ni.2419>
- Geissmann, F., Jung, S., & Littman, D. R. (2003). Blood monocytes consist of two principal subsets with distinct migratory properties. *Immunity*, 19(1), 71–82. [https://doi.org/10.1016/s1074-7613\(03\)00174-2](https://doi.org/10.1016/s1074-7613(03)00174-2)
- Geissmann, F., Manz, M. G., Jung, S., Sieweke, M. H., Merad, M., & Ley, K. (2010). Development of monocytes, macrophages, and dendritic cells. *Science*, 327(5966), 656–661. <https://doi.org/10.1126/science.1178331>
- Ginhoux, F., Greter, M., Leboeuf, M., Nandi, S., See, P., Gokhan, S., Mehler, M. F., Conway, S. J., Ng, L. G., Stanley, E. R., Samokhvalov, I. M., & Merad, M. (2010). Fate mapping analysis reveals that adult microglia derive from primitive macrophages. *Science*, 330(6005), 841–845. <https://doi.org/10.1126/science.1194637>
- Goldman, T., Wieghofer, P., Jordão, M. J. C., Prutek, F., Hagemeyer, N., Frenzel, K., Amann, L., Staszewski, O., Kierdorf, K., Krueger, M., Locatelli, G., Hochgerner, H., Zeiser, R., Epelman, S., Geissmann, F., Priller, J., Rossi, F. M. V., Bechmann, I., Kerschensteiner, M., ... Prinz, M. (2016). Origin, fate and dynamics of macrophages at central nervous system interfaces. *Nature Immunology*, 17(7), 797–805. <https://doi.org/10.1038/ni.3423>
- Gomez Perdiguero, E., Klapproth, K., Schulz, C., Busch, K., Azzoni, E., Crozet, L., Garner, H., Trouillet, C., de Bruijn, M. F., Geissmann, F., & Rodewald, H.-R. (2015). Tissue-resident macrophages originate from yolk-sac-derived erythro-myeloid progenitors. *Nature*, 518(7540), 547–551. <https://doi.org/10.1038/nature13989>
- Gosselin, D., Link, V. M., Romanoski, C. E., Fonseca, G. J., Eichenfield, D. Z., Spann, N. J., Stender, J. D., Chun, H. B., Garner, H., Geissmann, F., & Glass, C. K. (2014). Environment drives selection and function of enhancers controlling tissue-specific macrophage identities. *Cell*, 159(6), 1327–1340. <https://doi.org/10.1016/j.cell.2014.11.023>
- Grubman, A., Choo, X. Y., Chew, G., Ouyang, J. F., Sun, G., Croft, N. P., Rossello, F. J., Simmons, R., Buckberry, S., Landin, D. V., Pflueger, J., Vandekolk, T. H., Abay, Z., Zhou, Y., Liu, X., Chen, J., Larcombe, M., Haynes, J. M., McLean, C., ... Polo, J. M. (2021). Transcriptional signature in microglia associated with Aβ plaque phagocytosis. *Nature Communications*, 12(1), 3015. <https://doi.org/10.1038/s41467-021-23111-1>
- Gschwandtner, M., Derler, R., & Midwood, K. S. (2019). More than just attractive: How CCL2 influences myeloid cell behavior beyond chemotaxis. *Frontiers in Immunology*, 10, 2759. <https://doi.org/10.3389/fimmu.2019.02759>
- Guerreiro, R., Wojtas, A., Bras, J., Carrasquillo, M., Rogava, E., Majounie, E., Cruchaga, C., Sassi, C., Kauwe, J. S., Younkin, S., Hazrati, L., Collinge, J., Pocock, J., Lashley, T., Williams, J.,

- Lambert, J. C., Amouyel, P., Goate, A., Rademakers, R., ... Hardy, J. (2013). TREM2 variants in Alzheimer's disease. *The New England Journal of Medicine*, 368(2), 117–127. <https://doi.org/10.1056/NEJMoa1211851>
- Haage, V., Semtner, M., Vidal, R. O., Hernandez, D. P., Pong, W. W., Chen, Z., Hambardzumyan, D., Magrini, V., Ly, A., Walker, J., Mardis, E., Mertins, P., Sauer, S., Kettenmann, H., & Gutmann, D. H. (2019). Comprehensive gene expression meta-analysis identifies signature genes that distinguish microglia from peripheral monocytes/macrophages in health and glioma. *Acta Neuropathologica Communications*, 7(1), 20. <https://doi.org/10.1186/s40478-019-0665-y>
- Hammond, T. R., Dufort, C., Dissing-Olesen, L., Giera, S., Young, A., Wysoker, A., Walker, A. J., Gergits, F., Segel, M., Nemes, J., Marsh, S. E., Saunders, A., Macosko, E., Ginhoux, F., Chen, J., Franklin, R. J. M., Piao, X., McCarroll, S. A., & Stevens, B. (2019). Single-cell RNA sequencing of microglia throughout the mouse lifespan and in the injured brain reveals complex cell-state changes. *Immunity*, 50(1), 253–271.e256. <https://doi.org/10.1016/j.immuni.2018.11.004>
- Hawkes, C. A., & McLaurin, J. (2009). Selective targeting of perivascular macrophages for clearance of beta-amyloid in cerebral amyloid angiopathy. *Proceedings of the National Academy of Sciences of the United States of America*, 106(4), 1261–1266. <https://doi.org/10.1073/pnas.0805453106>
- Held, K. S., Chen, B. P., Kuziel, W. A., Rollins, B. J., & Lane, T. E. (2004). Differential roles of CCL2 and CCR2 in host defense to coronavirus infection. *Virology*, 329(2), 251–260. <https://doi.org/10.1016/j.virol.2004.09.006>
- Herz, J., Filiano, A. J., Smith, A., Yogev, N., & Kipnis, J. (2017). Myeloid cells in the central nervous system. *Immunity*, 46(6), 943–956. <https://doi.org/10.1016/j.immuni.2017.06.007>
- Hohsfield, L. A., Najafi, A. R., Ghorbanian, Y., Soni, N., Hingco, E. E., Kim, S. J., Jue, A. D., Swarup, V., Inlay, M. A., & Green, K. N. (2020). Effects of long-term and brain-wide colonization of peripheral bone marrow-derived myeloid cells in the CNS. *Journal of Neuroinflammation*, 17(1), 279. <https://doi.org/10.1186/s12974-020-01931-0>
- Holtman, I. R., Raj, D. D., Miller, J. A., Schaafsma, W., Yin, Z., Brouwer, N., Wes, P. D., Möller, T., Orre, M., Kamphuis, W., Hol, E. M., Boddeke, E. W. G. M., & Eggen, B. J. (2015). Induction of a common microglia gene expression signature by aging and neurodegenerative conditions: A co-expression meta-analysis. *Acta Neuropathologica Communications*, 3, 31. <https://doi.org/10.1186/s40478-015-0203-5>
- Honaripisheh, P., Lee, J., Banerjee, A., Blasco-Conesa, M. P., Honaripisheh, P., d'Aigle, J., Mamun, A. A., Ritzel, R. M., Chauhan, A., Ganesh, B. P., & McCullough, L. D. (2020). Potential caveats of putative microglia-specific markers for assessment of age-related cerebrovascular neuroinflammation. *Journal of Neuroinflammation*, 17(1), 366. <https://doi.org/10.1186/s12974-020-02019-5>
- Hosking, M. P., & Lane, T. E. (2010). The role of chemokines during viral infection of the CNS. *PLoS Pathogens*, 6(7), e1000937. <https://doi.org/10.1371/journal.ppat.1000937>
- Jay, T. R., Miller, C. M., Cheng, P. J., Graham, L. C., Bemiller, S., Broihier, M. L., Xu, G., Margevicius, D., Karlo, J. C., Sousa, G. L., Cotleur, A. C., Butovsky, O., Bekris, L., Staugaitis, S. M., Leverenz, J. B., Pimprikar, S. W., Landreth, G. E., Howell, G. R., Ransohoff, R. M., & Lamb, B. T. (2015). TREM2 deficiency eliminates TREM2+ inflammatory macrophages and ameliorates pathology in Alzheimer's disease mouse models. *The Journal of Experimental Medicine*, 212(3), 287–295. <https://doi.org/10.1084/jem.20142322>
- Jonsson, T., Stefansson, H., Steinberg, S., Jonsson, P. V., Snaedal, J., Bjornsson, S., Huttenlocher, J., Levey, A. I., Lah, J. J., Rujescu, D., Hampel, H., Giegling, I., Andreassen, O. A., Engedal, K., Ulstein, I., Djurovic, S., Ibrahim-Verbaas, C., Hofman, A., ... Stefansson, K. (2013). Variant of TREM2 associated with the risk of Alzheimer's disease. *The New England Journal of Medicine*, 368(2), 107–116. <https://doi.org/10.1056/NEJMoa1211103>
- Jordao, M. J. C., Sankowski, R., Brendecke, S. M., Sagar, Locatelli, G., Tai, Y. H., Tay, T. L., Schramm, E., Armbruster, S., Hagemeyer, N., Groß, O., Mai, D., Çiçek, Ö., Falk, T., Kerschensteiner, M., Grün, D., & Prinz, M. (2019). Single-cell profiling identifies myeloid cell subsets with distinct fates during neuroinflammation. *Science*, 363(6425), eaat7554. <https://doi.org/10.1126/science.aat7554>
- Jung, S., Aliberti, J., Graemmel, P., Sunshine, M. J., Kreutzberg, G. W., Sher, A., & Littman, D. R. (2000). Analysis of fractalkine receptor CX3CR1 function by targeted deletion and green fluorescent protein reporter gene insertion. *Molecular and Cellular Biology*, 20(11), 4106–4114. <https://doi.org/10.1128/mcb.20.11.4106-4114.2000>
- Keren-Shaul, H., Spinrad, A., Weiner, A., Matcovitch-Natan, O., Dvir-Szternfeld, R., Ulland, T. K., David, E., Baruch, K., Lara-Astaiso, D., Toth, B., Itzkovitz, S., Colonna, M., Schwartz, M., & Amit, I. (2017). A unique microglia type associated with restricting development of Alzheimer's disease. *Cell*, 169(7), 1276–1290.e1217. <https://doi.org/10.1016/j.cell.2017.05.018>
- Kierdorf, K., Erny, D., Goldmann, T., Sander, V., Schulz, C., Perdiguero, E. G., Wieghofer, P., Heinrich, A., Riemke, P., Hölscher, C., Müller, D. N., Luckow, B., Bockler, T., Debowski, K., Fritz, G., Opendakker, G., Diefenbach, A., Biber, K., Heikenwalder, M., ... Prinz, M. (2013). Microglia emerge from erythromyeloid precursors via Pu.1- and Irf8-dependent pathways. *Nature Neuroscience*, 16(3), 273–280. <https://doi.org/10.1038/nn.3318>
- Kierdorf, K., Masuda, T., Jordão, M. J. C., & Prinz, M. (2019). Macrophages at CNS interfaces: Ontogeny and function in health and disease. *Nature Reviews Neuroscience*, 20(9), 547–562. <https://doi.org/10.1038/s41583-019-0201-x>
- Konishi, H., Kobayashi, M., Kunisawa, T., Imai, K., Sayo, A., Malissen, B., Crocker, P. R., Sato, K., & Kiyama, H. (2017). Siglec-H is a microglia-specific marker that discriminates microglia from CNS-associated macrophages and CNS-infiltrating monocytes. *Glia*, 65(12), 1927–1943. <https://doi.org/10.1002/glia.23204>
- Korin, B., Ben-Shaanan, T. L., Schiller, M., Dubovik, T., Azulay-Debby, H., Boshnak, N. T., Koren, T., & Rolls, A. (2017). High-dimensional, single-cell characterization of the brain's immune compartment. *Nature Neuroscience*, 20(9), 1300–1309. <https://doi.org/10.1038/nn.4610>
- Krasemann, S., Madore, C., Cialic, R., Baufeld, C., Calcagno, N., el Fatimy, R., Beckers, L., O'Loughlin, E., Xu, Y., Fanek, Z., Greco, D. J., Smith, S. T., Tweet, G., Humulock, Z., Zrzavy, T., Conde-Sanroman, P., Gacias, M., Weng, Z., Chen, H., ... Butovsky, O. (2017). The TREM2-APOE pathway drives the transcriptional phenotype of dysfunctional microglia in neurodegenerative diseases. *Immunity*, 47(3), 566–581.e569. <https://doi.org/10.1016/j.immuni.2017.08.008>
- Lambert, J. C., Ibrahim-Verbaas, C. A., Harold, D., Naj, A. C., Sims, R., Bellenguez, C., Jun, G., DeStefano, A. L., Bis, J. C., Beecham, G. W., Grenier-Boley, B., Russo, G., Thornton-Wells, T. A., Jones, N., Smith, A. V., Chouraki, V., Thomas, C., Ikram, M. A., Zelenika, D., ... Amouyel, P. (2013). Meta-analysis of 74,046 individuals identifies 11 new susceptibility loci for Alzheimer's disease. *Nature Genetics*, 45(12), 1452–1458. <https://doi.org/10.1038/ng.2802>
- Lampert, P. W., Sims, J. K., & Kniatzeff, A. J. (1973). Mechanism of demyelination in JHM virus encephalomyelitis. Electron microscopic studies. *Acta neuropathologica*, 24(1), 76–85. <https://doi.org/10.1007/bf00691421>
- Lane, T. E., & Buchmeier, M. J. (1997). Murine coronavirus infection: A paradigm for virus-induced demyelinating disease. *Trends in Microbiology*, 5(1), 9–14. [https://doi.org/10.1016/s0966-842x\(97\)81768-4](https://doi.org/10.1016/s0966-842x(97)81768-4)
- Lane, T. E., & Hosking, M. P. (2010). The pathogenesis of murine coronavirus infection of the central nervous system. *Critical Reviews in Immunology*, 30(2), 119–130. <https://doi.org/10.1615/critrevimmunol.v30.i2.20>
- Lavin, Y., Winter, D., Blecher-Gonen, R., David, E., Keren-Shaul, H., Merad, M., Jung, S., & Amit, I. (2014). Tissue-resident macrophage





- enhancer landscapes are shaped by the local microenvironment. *Cell*, 159(6), 1312–1326. <https://doi.org/10.1016/j.cell.2014.11.018>
- Li, Q., Cheng, Z., Zhou, L., Darmanis, S., Neff, N. F., Okamoto, J., Gulati, G., Bennett, M. L., Sun, L. O., Clarke, L. E., Marschallinger, J., Yu, G., Quake, S. R., Wyss-Coray, T., & Barres, B. A. (2019). Developmental heterogeneity of microglia and brain myeloid cells revealed by deep single-cell RNA sequencing. *Neuron*, 101(2), 207–223.e210. <https://doi.org/10.1016/j.neuron.2018.12.006>
- Liu, Z., Gu, Y., Chakarov, S., Bleriot, C., Kwok, I., Chen, X., Shin, A., Huang, W., Dress, R. J., Dutertre, C. A., Schlitzer, A., Chen, J., Ng, L. G., Wang, H., Liu, Z., Su, B., & Ginhoux, F. (2019). Fate mapping via Ms4a3-expression history traces monocyte-derived cells. *Cell*, 178(6), 1509–1525.e1519. <https://doi.org/10.1016/j.cell.2019.08.009>
- Lund, H., Pieber, M., Parsa, R., Han, J., Grommisch, D., Ewing, E., Kular, L., Needhamsen, M., Espinosa, A., Nilsson, E., Överby, A. K., Butovsky, O., Jagodic, M., Zhang, X. M., & Harris, R. A. (2018). Competitive repopulation of an empty microglial niche yields functionally distinct subsets of microglia-like cells. *Nature Communications*, 9(1), 4845. <https://doi.org/10.1038/s41467-018-07295-7>
- MacKinnon, A. C., Farnworth, S. L., Hodkinson, P. S., Henderson, N. C., Atkinson, K. M., Leffler, H., Nilsson, U. J., Haslett, C., Forbes, S. J., & Sethi, T. (2008). Regulation of alternative macrophage activation by galectin-3. *Journal of Immunology (Baltimore, Md.: 1950)*, 180(4), 2650–2658. <https://doi.org/10.4049/jimmunol.180.4.2650>
- Marro, B. S., Grist, J. J., & Lane, T. E. (2016). Inducible expression of CXCL1 within the central nervous system amplifies viral-induced demyelination. *Journal of Immunology*, 196(4), 1855–1864. <https://doi.org/10.4049/jimmunol.1501802>
- Marsh, S. E., Kamath, T., Walker, A. J., Dissing-Olesen, L., Hammond, T. R., Young, A. M. H., Abdulraouf, A., Nadaf, N., Dufort, C., Murphy, S., Kozareva, V., Vanderburg, C., Hong, S., Bulstrode, H., Hutchinson, P. J., Gaffney, D. J., Franklin, R. J. M., Macosko, E. Z., & Stevens, B. (2020). Single cell sequencing reveals glial specific responses to tissue processing & enzymatic dissociation in mice and humans. *BioRxiv*. <https://doi.org/10.1101/2020.12.03.408542>
- Masuda, T., Sankowski, R., Staszewski, O., Bottcher, C., Amann, L., Scheiwe, C., Nessler, S., Kunz, P., van Loo, G., Coenen, V. A., Reinacher, P. C., Michel, A., Sure, U., Gold, R., Grün, D., Priller, J., Stadelmann, C., & Prinz, M. (2019). Spatial and temporal heterogeneity of mouse and human microglia at single-cell resolution. *Nature*, 566(7744), 388–392. <https://doi.org/10.1038/s41586-019-0924-x>
- Mehina, E. M. F., Taylor, S., Boghozian, R., White, E., Choi, S. E., Cheema, M. S., Korbelen, J., & Brown, C. E. (2021). Invasion of phagocytic galectin 3 expressing macrophages in the diabetic brain disrupts vascular repair. *Science Advances*, 7(34), eabg2712. <https://doi.org/10.1126/sciadv.abg2712>
- Mildner, A., Schmidt, H., Nitsche, M., Merkler, D., Hanisch, U. K., Mack, M., Heikenwalder, M., Brück, W., Priller, J., & Prinz, M. (2007). Microglia in the adult brain arise from Ly-6ChiCCR2+ monocytes only under defined host conditions. *Nature Neuroscience*, 10(12), 1544–1553. <https://doi.org/10.1038/nn2015>
- Mildner, A., Schönheit, J., Giladi, A., David, E., Lara-Astiaso, D., Lorenzo-Vivas, E., Paul, F., Chappell-Maor, L., Priller, J., Leutz, A., Amit, I., & Jung, S. (2017). Genomic characterization of murine monocytes reveals C/EBP $\beta$  transcription factor dependence of Ly6C(–) cells. *Immunity*, 46(5), 849–862.e847. <https://doi.org/10.1016/j.immuni.2017.04.018>
- Mrdjen, D., Pavlovic, A., Hartmann, F. J., Schreiner, B., Utz, S. G., Leung, B. P., Lelios, I., Heppner, F. L., Kipnis, J., Merkler, D., Greter, M., & Becher, B. (2018). High-dimensional single-cell mapping of central nervous system immune cells reveals distinct myeloid subsets in health, aging, and disease. *Immunity*, 48(2), 380–395.e386. <https://doi.org/10.1016/j.immuni.2018.01.011>
- Munro, D. A. D., Bradford, B. M., Mariani, S. A., Hampton, D. W., Vink, C. S., Chandran, S., Hume, D. A., Pridans, C., & Priller, J. (2020). CNS macrophages differentially rely on an intronic Csf1r enhancer for their development. *Development*, 147(23), dev194449. <https://doi.org/10.1242/dev.194449>
- Oakley, H., Cole, S. L., Logan, S., Maus, E., Shao, P., Craft, J., Guillozet-Bongarts, A., Ohno, M., Disterhoft, J., van Eldik, L., Berry, R., & Vassar, R. (2006). Intraneuronal beta-amyloid aggregates, neurodegeneration, and neuron loss in transgenic mice with five familial Alzheimer's disease mutations: Potential factors in amyloid plaque formation. *The Journal of Neuroscience*, 26(40), 10129–10140. <https://doi.org/10.1523/jneurosci.1202-06.2006>
- Ochocka, N., Segit, P., Walentynowicz, K. A., Wojnicki, K., Cyranowski, S., Swatler, J., Mieczkowski, J., & Kaminska, B. (2021). Single-cell RNA sequencing reveals functional heterogeneity of glioma-associated brain macrophages. *Nature Communications*, 12(1), 1151. <https://doi.org/10.1038/s41467-021-21407-w>
- Prinz, M., & Priller, J. (2014). Microglia and brain macrophages in the molecular age: From origin to neuropsychiatric disease. *Nature Reviews. Neuroscience*, 15(5), 300–312. <https://doi.org/10.1038/nrn3722>
- Prinz, M., Priller, J., Sisodia, S. S., & Ransohoff, R. M. (2011). Heterogeneity of CNS myeloid cells and their roles in neurodegeneration. *Nature Neuroscience*, 14(10), 1227–1235. <https://doi.org/10.1038/nn.2923>
- Ransohoff, R. M., & Cardona, A. E. (2010). The myeloid cells of the central nervous system parenchyma. *Nature*, 468(7321), 253–262. <https://doi.org/10.1038/nature09615>
- Reed-Geaghan, E. G., Croxford, A. L., Becher, B., & Landreth, G. E. (2020). Plaque-associated myeloid cells derive from resident microglia in an Alzheimer's disease model. *The Journal of Experimental Medicine*, 217(4), e20191374. <https://doi.org/10.1084/jem.20191374>
- Rojo, R., Sauter, K. A., Lefevre, L., Hume, D. A., & Pridans, C. (2018). Maternal tamoxifen treatment expands the macrophage population of early mouse embryos. *bioRxiv*, 296749. <https://doi.org/10.1101/296749>
- Saederup, N., Cardona, A. E., Croft, K., Mizutani, M., Cotleur, A. C., Tsou, C. L., Ransohoff, R. M., & Charo, I. F. (2010). Selective chemokine receptor usage by central nervous system myeloid cells in CCR2-red fluorescent protein knock-in mice. *PLoS One*, 5(10), e13693. <https://doi.org/10.1371/journal.pone.0013693>
- Safaiyan, S., Besson-Girard, S., Kaya, T., Cantuti-Castelvetri, L., Liu, L., Ji, H., Schifferer, M., Gouna, G., Usifo, F., Kannaiyan, N., Fitzner, D., Xiang, X., Rossner, M. J., Brendel, M., Gokce, O., & Simons, M. (2021). White matter aging drives microglial diversity. *Neuron*, 109(7), 1100–1117.e1110. <https://doi.org/10.1016/j.neuron.2021.01.027>
- Savarin, C., Stohlman, S. A., Atkinson, R., Ransohoff, R. M., & Bergmann, C. C. (2010). Monocytes regulate T cell migration through the glia limitans during acute viral encephalitis. *Journal of Virology*, 84(10), 4878–4888. <https://doi.org/10.1128/jvi.00051-10>
- Shemer, A., Erny, D., Jung, S., & Prinz, M. (2015). Microglia plasticity during health and disease: An immunological perspective. *Trends in Immunology*, 36(10), 614–624. <https://doi.org/10.1016/j.it.2015.08.003>
- Shemer, A., Grozovski, J., Tay, T. L., Tao, J., Volaski, A., Süß, P., Ardura-Fabregat, A., Gross-Vered, M., Kim, J. S., David, E., Chappell-Maor, L., Thielecke, L., Glass, C. K., Cornils, K., Prinz, M., & Jung, S. (2018). Engrafted parenchymal brain macrophages differ from microglia in transcriptome, chromatin landscape and response to challenge. *Nature Communications*, 9(1), 5206. <https://doi.org/10.1038/s41467-018-07548-5>
- Shukla, A. K., McIntyre, L. L., Marsh, S. E., Schneider, C. A., Hoover, E. M., Walsh, C. M., Lodoen, M. B., Blurton-Jones, M., & Inlay, M. A. (2019). CD11a expression distinguishes infiltrating myeloid cells from plaque-associated microglia in Alzheimer's disease. *Glia*, 67(5), 844–856. <https://doi.org/10.1002/glia.23575>
- Simard, A. R., Soulet, D., Gowing, G., Julien, J. P., & Rivest, S. (2006). Bone marrow-derived microglia play a critical role in restricting senile plaque formation in Alzheimer's disease. *Neuron*, 49(4), 489–502. <https://doi.org/10.1016/j.neuron.2006.01.022>

- Sobue, A., Komine, O., Hara, Y., Endo, F., Mizoguchi, H., Watanabe, S., Murayama, S., Saito, T., Saido, T. C., Sahara, N., Higuchi, M., Ogi, T., & Yamanaka, K. (2021). Microglial gene signature reveals loss of homeostatic microglia associated with neurodegeneration of Alzheimer's disease. *Acta Neuropathologica Communications*, 9(1), 1. <https://doi.org/10.1186/s40478-020-01099-x>
- Spangenberg, E., Severson, P. L., Hohsfield, L. A., Crapser, J., Zhang, J., Burton, E. A., Zhang, Y., Spevak, W., Lin, J., Phan, N. Y., Habets, G., Rymar, A., Tsang, G., Walters, J., Nespi, M., Singh, P., Broome, S., Ibrahim, P., Zhang, C., ... Green, K. N. (2019). Sustained microglial depletion with CSF1R inhibitor impairs parenchymal plaque development in an Alzheimer's disease model. *Nature Communications*, 10(1), 3758. <https://doi.org/10.1038/s41467-019-11674-z>
- Steinbach, F., & Thiele, B. (1994). Phenotypic investigation of mononuclear phagocytes by flow cytometry. *Journal of Immunological Methods*, 174(1–2), 109–122. [https://doi.org/10.1016/0022-1759\(94\)90015-9](https://doi.org/10.1016/0022-1759(94)90015-9)
- Syage, A. R., Ekiz, H. A., Skinner, D. D., Stone, C., O'Connell, R. M., & Lane, T. E. (2020). Single-cell RNA sequencing reveals the diversity of the immunological landscape following central nervous system infection by a murine coronavirus. *Journal of Virology*, 94(24), e01295. <https://doi.org/10.1128/jvi.01295-20>
- Thomas, L., & Pasquini, L. A. (2018). Galectin-3-mediated glial crosstalk drives oligodendrocyte differentiation and (re)myelination. *Frontiers in Cellular Neuroscience*, 12, 297. <https://doi.org/10.3389/fncel.2018.00297>
- Utz, S. G., See, P., Mildenerger, W., Thion, M. S., Silvin, A., Lutz, M., Ingelfinger, F., Rayan, N. A., Lelios, I., Buttgerit, A., Asano, K., Prabhakar, S., Garel, S., Becher, B., Ginhoux, F., & Greter, M. (2020). Early fate defines microglia and non-parenchymal brain macrophage development. *Cell*, 181(3), 557–573.e518. <https://doi.org/10.1016/j.cell.2020.03.021>
- van Hove, H., Martens, L., Scheyltjens, I., de Vlaminc, K., Pombo Antunes, A. R., de Prijk, S., Vandamme, N., de Schepper, S., van Isterdael, G., Scott, C. L., Aerts, J., Berx, G., Boeckxstaens, G. E., Vandenbroucke, R. E., Vereecke, L., Moechars, D., Guillems, M., van Ginderachter, J. A., Saeys, Y., & Movahedi, K. (2019). A single-cell atlas of mouse brain macrophages reveals unique transcriptional identities shaped by ontogeny and tissue environment. *Nature Neuroscience*, 22(6), 1021–1035. <https://doi.org/10.1038/s41593-019-0393-4>
- Wahane, S., Zhou, X., Zhou, X., Guo, L., Friedl, M.-S., Kluge, M., Ramakrishnan, A., Shen, L., Friedel, C. C., Zhang, B., Friedel, R. H., & Zou, H. (2021). Diversified transcriptional responses of myeloid and glial cells in spinal cord injury shaped by HDAC3 activity. *Science Advances*, 7(9), eabd8811. <https://doi.org/10.1126/sciadv.abd8811>
- Wang, Y., Ulland, T. K., Ulrich, J. D., Song, W., Tzaferis, J. A., Hole, J. T., Yuan, P., Mahan, T. E., Shi, Y., Gilfillan, S., Cella, M., Grutzendler, J., DeMattos, R. B., Cirrito, J. R., Holtzman, D. M., & Colonna, M. (2016). TREM2-mediated early microglial response limits diffusion and toxicity of amyloid plaques. *The Journal of Experimental Medicine*, 213(5), 667–675. <https://doi.org/10.1084/jem.20151948>
- Weiner, L. P. (1973). Pathogenesis of demyelination induced by a mouse hepatitis. *Archives of Neurology*, 28(5), 298–303. <https://doi.org/10.1001/archneur.1973.00490230034003>
- Werner, Y., Mass, E., Ashok Kumar, P., Ulas, T., Händler, K., Horne, A., Klee, K., Lupp, A., Schütz, D., Saaber, F., Redecker, C., Schultze, J. L., Geissmann, F., & Stumm, R. (2020). Cxcr4 distinguishes HSC-derived monocytes from microglia and reveals monocyte immune responses to experimental stroke. *Nature Neuroscience*, 23(3), 351–362. <https://doi.org/10.1038/s41593-020-0585-y>
- Wong, L. M., Myers, S. J., Tsou, C. L., Gosling, J., Arai, H., & Charo, I. F. (1997). Organization and differential expression of the human monocyte chemoattractant protein 1 receptor gene. Evidence for the role of the carboxyl-terminal tail in receptor trafficking. *The Journal of Biological Chemistry*, 272(2), 1038–1045. <https://doi.org/10.1074/jbc.272.2.1038>
- Yamasaki, R., Lu, H., Butovsky, O., Ohno, N., Rietsch, A. M., Cialic, R., Wu, P. M., Doykan, C. E., Lin, J., Cottle, A. C., Kidd, G., Zorlu, M. M., Sun, N., Hu, W., Liu, L. P., Lee, J. C., Taylor, S. E., Uehlein, L., Dixon, D., ... Ransohoff, R. M. (2014). Differential roles of microglia and monocytes in the inflamed central nervous system. *The Journal of Experimental Medicine*, 211(8), 1533–1549. <https://doi.org/10.1084/jem.20132477>
- Zhan, L., Fan, L., Kodama, L., Sohn, P. D., Wong, M. Y., Mousa, G. A., Zhou, Y., Li, Y., & Gan, L. (2020). A MAC2-positive progenitor-like microglial population is resistant to CSF1R inhibition in adult mouse brain. *eLife*, 9, e51796. <https://doi.org/10.7554/eLife.51796>

**How to cite this article:** Hohsfield, L. A., Tsourmas, K. I., Ghorbanian, Y., Syage, A. R., Jin Kim, S., Cheng, Y., Furman, S., Inlay, M. A., Lane, T. E., & Green, K. N. (2022). MAC2 is a long-lasting marker of peripheral cell infiltrates into the mouse CNS after bone marrow transplantation and coronavirus infection. *Glia*, 1–17. <https://doi.org/10.1002/glia.24144>