UNDERSTANDING THE ROLES OF GLIA AND CIRCULATING LEUKOCYTES IN NEURODEGENERATIVE DISEASES

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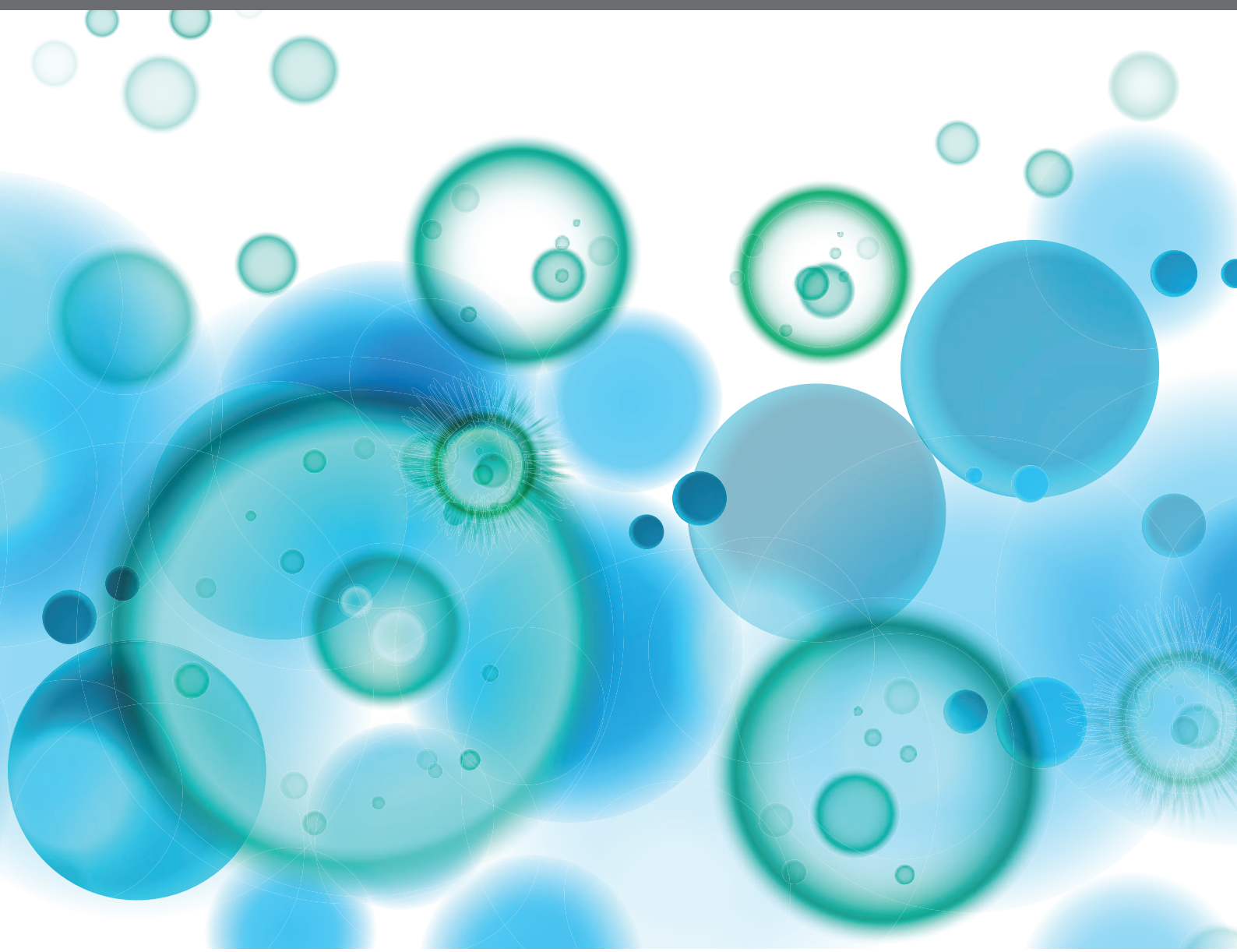

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UNDERSTANDING THE ROLES OF GLIA AND CIRCULATING LEUKOCYTES IN NEURODEGENERATIVE DISEASES

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Role of Macrophage Colony-Stimulating Factor Receptor on the Proliferation and Survival of Microglia Following Systemic Nerve and Cuprizone-Induced Injuries

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Microglia are the innate immune cells of the CNS and their proliferation, activation, and survival have previously been shown to be highly dependent on macrophage colony-stimulating factor receptor (CSF1R). Here we investigated the impact of the receptor in such processes using two different models of nerve injuries, namely hypoglossal axotomy and cuprizone-induced demyelination. Both models are associated with a robust microgliosis. The role of CSF1R was investigated using the gene deletion Cre/Lox system, which allows the conditional knock-out following tamoxifen administration. We found that after 5 weeks of cuprizone diet that CSF1R suppression caused a significant impairment of microglia function. A reduced microgliosis was detected in the corpus collosum of CSF1R knock-out mice compared to controls. In contrast to cuprizone model, the overall number of Iba1 cells was unchanged at all the times evaluated following hypoglossal axotomy in WT and cKO conditions. After nerve lesion, a tremendous proliferation was noticed in the ipsilateral hypoglossal nucleus to a similar level in both knock-out and wild-type groups. We also observed infiltration of bone-marrow derived cells specifically in CSF1R-deficient mice, these cells tend to compensate the CSF1R signaling pathway suppression in resident microglia. Taking together our results suggest a different role of CSF1R in microglia depending on the model. In the pathologic context of cuprizone-induced demyelination CSF1R signaling pathway is essential to trigger proliferation and survival of microglia, while this is not the case in a model of systemic nerve injury. M-CSF/CSF1R is consequently not the unique system involved in microgliosis following nerve damages.

Keywords: microglia, proliferation, brain injuries, demyelination, monocytes, CSF1R

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INTRODUCTION

Macrophage colony-stimulating factor receptor (CSF1R) is a receptor of the tyrosine kinase family. It is broadly expressed in the organism by monocytes, resident macrophages, osteoclasts, Paneth cells, dendritic cells, and in the brain microglia. Two ligands bind CSF1R, macrophage colony-stimulating factor (mCSF) and Interleukine-34 (IL-34), which have complementary roles on the

proliferation of innate immune cells, especially on monocyte and macrophage populations (1). In the brain, it acts on the phagocytosis, survival, and proliferation of microglia (2). Microglia are the resident immune cells of the brain. These mononuclear phagocytes arise from hematopoietic progenitors in the yolk sac during embryogenesis and are generated at the postnatal stage. These immune cells are implicated in brain homeostasis, and can detect any inflammatory and damage sites (3).

Microglia and CSF1R signaling pathway are involved in different neuroprotective roles, such as clearing myelin debris and toxic proteins from the cerebral environment. In this regard, modulation of the receptor is thought to be a novel therapeutic avenue for diseases, such as Alzheimer's disease (AD), multiple sclerosis (MS), and brain tumors (2, 4–7). To better understand how CSF1R drives proliferation of microglial cells, we have deleted this receptor specifically in CX3CR1-postive cells in different nerve damage and pathologic models. Hypoglossal axotomy model provides a sterile proliferative system to better understand the cellular and molecular events associated with microglia proliferation, without interfering with the environment found from pathological models (8). After the systemic nerve section, a tremendous microglial proliferation takes place in the ipsilateral hypoglossal nucleus. The cuprizone model allows to study cellular and molecular mechanisms involved in the demyelination/remyelination processes, while excluding the autoimmune component. Here also there is a robust microgliosis in brain regions where myelin and oligodendrocytes are affected by the copper-chelating toxin (9).

Here we have investigated the role of CSF1R on the proliferation of microglia using the gene deletion Cre/Lox system, which allows the knock-out following tamoxifen administration. We used two different models causing microglial cell proliferation to determine whether CSF1R is crucial for both sterile and toxin cues. Our results show that CSF1R is essential for microglia proliferation in cuprizone-fed mice indicating that this signaling pathway in such a mouse model of progressing MS is vital for microglial survival and proliferation. Surprisingly deletion of this receptor has no impact on such cellular functions following hypoglossal nerve injury. Moreover, a compensatory mechanism seems to take place for the loss of CSF1R with overexpression of TREM2 in microglia.

MATERIALS AND METHODS

Animals Surgery

Animals were injected with tamoxifen following several time-course (**Figure 2**). Males, 2 to 4-month-old mice were anesthetized using isofluorane (Baxter Corporation, Ontario, Canada) 3–4% and oxygen 0.8–1.5 l/min, then they were shaved. To mitigate the pain, we used Maxilene®4, lidocaine cream 4% (Ferndale Laboratories, Inc., MI 48220 USA), applied on the neck 5 min prior the surgery. After the pre-surgery preparation, isofluorane is set to 1.5–2% and oxygen flux was adjusted to 1.5–2 l/min. Animals were placed in the supine position, and the right hypoglossal nerve was transected with scissors. Mice were kept alive 1 week after surgery.

Cuprizone Diet

0.2% wt/wt cuprizone (bis-cyclohexylidene hydrazide; Sigma-Aldrich) was mixed with regular ground irradiated chow and fed to experimental animals for 5 weeks. The chow was changed every 2 days and food intake was monitored throughout the protocols. Control animals were fed with regular irradiated ground chow and manipulated as often as cuprizone-fed mice. After terminating the 5 weeks of cuprizone diet mice were euthanized.

Conditional CSF1R KO Mice

B6.Cg-Csf1r *tm1jwp*/J mice (JaxMice; stock number 02212) were crossed with the B6.129-Cx3cr1tm2.1 (CreER)Jung/Orl mice (EMMA mouse respiratory; EM:06350). The resulting mouse has a tamoxifen-inducible CRE activity specifically in microglial cells, leading to a non-functional CRF1R protein.

Tamoxifen Preparation and Administration

Tamoxifen was dissolved in corn oil and Ethanol 100% for 1 h at 37 degrees, vortexed every 15 min. We used ∼75 mg tamoxifen/Kg body weight and 100 µl tamoxifen/corn oil solution was administered via intraperitoneal injection for 4 consecutive days. Tamoxifen was injected 4 days before cuprizone diet, and 7, 13, or 21 days before nerve transection. For the hypoglossal nerve lesion, protocols are named, respectively, Short Protocol (SP), Principal protocol (P1), and Long Protocol (LP).

Chimeric Mice

Experimental animals received a total of 80 mg/kg of Busulfan administered i.p. every 12 h for 4 days, followed by 2 days of single i.p. injection of 100 mg/kg cyclophosphamide. After a 24-h rest, 3×10^7 bone marrow cells isolated from the tibia and femur of donor mice were injected into the tail vein of target animals. C57BL/6-Tg (CAG-EGFP) 1O sb/J (JaxMice stock number 003291) mice were used as donors. For details on this procedure, please refer to Laflamme et al. (10).

Sacrifices

All mice were deeply anesthetized with ketamine/xylazine and sacrificed via intracardiac perfusion with 0.9% saline followed by 4% PFA pH 7.4 or pH 9. The brains were then retrieved, post-fixed 10–24 h in 4% PFA pH 7.4 and transferred in 4% PFA pH 7.4 + 20% sucrose for a minimum of 15 h. Brains were sliced in coronal sections of 20-µm thickness with a freezing microtome (Leica Microsystems), serially collected in anti-freeze solution and kept at −20°C until usage.

Immunohistochemical Staining

Brain sections were washed (4 × 5 min) in KPBS. An antigen retrieval step was performed to stain for CSF1R. More specifically, sections were boiled 10 min in sodium citrate 10 mM pH 6 just before the blocking step and then blocked in KPBS containing 1% BSA, and 1% Triton X-100. The tissues were then incubated overnight at 4°C with the primary antibody anti-Iba-1 (rabbit, 1:1,000; WAKO Chemical 019-19741), or with the primary antibody anti-CSF1R (sheep, 1:500; R&D System AF3818), or with the primary antibody Tmem119 (rabbit, 1:1,000; ABCAM ab209064). After washing

the sections in KPBS (4 × 5 min), tissues were incubated in the appropriate secondary antibody (biotinylated goat anti-rabbit IgG; 1:1,500, Vector Laboratories. biotinylated rabbit anti-sheep IgG; 1:1,500, Vector Laboratories) for 2 h at room temperature. Following further washes in KPBS and 1 h-long incubation in avidin-biotin peroxidase complex (ABC; Vector Laboratories) the sections were then incubated in 3,3′-diaminobenzidine tetrahydrochloride (DAB; Sigma) to reveal the staining. The sections were mounted onto Micro Slides Superfrost® plus glass slides, dehydrated and then coverslipped with DPX mounting media.

Immunofluorescent Staining

Brain sections were washed (4 × 5 min) in KPBS then blocked in KPBS containing 1% BSA, and 1% Triton X-100. The tissues were then incubated overnight at 4°C with the primary antibody anti-Iba-1 (rabbit, 1:1,000; WAKO Chemical 019-19741). After washing the section in KPBS (4 × 5 min), the tissue was incubated in the appropriate secondary antibody (IgG anti-rabbit Alexa 546; Invitrogen A11010) for 2 h at room temperature. Following further washes in KPBS and incubation with DAPI to identify the nuclei, the sections were mounted onto Micro Slides Superfrost® Plus glass slides and coverslipped with Fluoromount-G (Electron Microscopy Sciences).

Western Blot

Brain protein lysates were extracted as previously described (11). Proteins were then loaded in 8–16% agarose precast gels (Biorad) and electroblotted onto 0.45 μm Immobilon PVDF membranes. Membranes were immunoblotted with primary antibodies anti-Iba-1 (rabbit, 1:1,000; WAKO Chemical 019-19741), followed by the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies and revealed by Clarity western (ECL) substrate (biorad). Quantification was done by determining integrative density of the bands using Thermo Scientific Pierce my Image Analysis Software v2.0. Optical values were normalized over actin.

Quantitative Real-Time PCR

Tissues were homogenized in Qiazol buffer (Qiagen, Germantown, MD, USA) and total RNA was extracted using the miRNeasy micro kit on-column DNase (Qiagen, Hilden, DE) treatment following the manufacturer's instructions. Quantity of total RNA was measured using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and total RNA quality was assayed on an Agilent BioAnalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA).

First-strand cDNA synthesis was accomplished using 4 μg of isolated RNA in a reaction containing 200 U of Superscript IV Rnase H-RT (Invitrogen Life Technologies, Burlington, ON, CA), 300 ng of oligo-dT_{18}, 50 ng of random hexamers, 50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 500 μM deoxynucleotides triphosphate, 5 mM dithiothreitol, and 40 U of Protector RNase inhibitor (Roche Diagnostics, Indianapolis, IN, USA) in a final volume of 50 μl. Reaction was incubated at 25°C for 10 min, then at 50°C for 20 min, inactivated at 80°C for 10 min. PCR purification kit (Qiagen, Hilden, DE) was used to purify cDNA.

Oligoprimer pairs were performed by IDT (Integrated DNA Technology, Coralville, IA, USA) (**Table 1**). A quantity corresponding to 20 ng of total RNA was used to perform fluorescent-based Realtime PCR quantification using the LightCycler 480 (Roche Diagnostics, Mannheim, DE). Reagent LightCycler 480 SYBRGreen I Master (Roche Diagnostics, Indianapolis, IN, USA) was used as described by the manufacturer with 2% DMSO. The conditions for PCR reactions were: 45 cycles, denaturation at 95°C for 10 s, annealing at 60°C for 10 s, elongation at 72°C for 14 s and then 74°C for 5 s (reading). A melting curve was performed to assess non-specific signal. Relative quantity was calculated using second derivative method and by applying the delta Ct (12). Normalization was performed using the reference gene shown to be genes having stable expression levels from embryonic life through adulthood in various tissues (13) hypoxanthine guanine phosphoribosyl transferase 1 (HPRT1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Quantitative Real-Time PCR measurements were performed by the CHU de Québec Research Center *(CHUL)* Gene Expression Platform, Quebec, Canada and were compliant with MIQE guidelines.

TABLE 1 | Sequence primers and gene description.

Gene symbol	Description	GenBank	Size (pb)	Primer sequence 5′ → 3′ S/AS
Tmem119	Mus musculus transmembrane protein 19 (Tmem19)	NM_133683	121	ACTGCTTCCTGGATGTGTTTGTCTC/ CCCAGGTTGTTATTAGCCGAGGT
TREM2	Mus musculus triggering receptor expressed on myeloid cells 2 (Trem2), 2 transcripts	NM_001272078	160	TGGTGTCGGCAGCTGGGTGAG/ CGGCTTGGAGGTTCTTCAGAGT
Hprt1	Mus musculus hypoxanthine guanine phosphoribosyl transferase 1	NM_013556	106	CAGGACTGAAAGACTTGCTCGAGAT/ CAGCAGGTCAGCAAAGAACTTATAGC
GAPDH	Mus musculus glyceraldehyde-3-phosphate dehydrogenase	NM_008084	194	ggctgcccagaacatcatccct/ atgcctgcttcaccaccttcttg
ADNg	Mus musculus chromosome 3 genomic contig, strain C57BL/6J (HSD3B1 intron)	NT_039239	209	CACCCCTTAAGAGACCCATGTT/ CCCTGCAGAGACCTTAGAAAAC

Image Acquisition and Analyses

Image acquisition of Fluorescent staining images was performed using a Zeiss LSM800 confocal microscope supported by the Zen software (2.3 system) using the 10×, 20×, and 40× lenses. Confocal images were then processed using Fiji (ImageJ Version 2.0.0-rc-43/1.51n). For analyses and brightfield image acquisition of staining, Iba-1 and CSF1R, 8-bit grayscale TIFF images of the regions of interest were taken in a single sitting for whole protocols with a Qimaging camera (Qcapture program, version 2.9.10), attached to Nikon microscope (C-80) with the same gain/exposure settings for every image. To evaluate the level of Iba-1$^+$ immune response in the regions of interest (corpus callosum and hypoglossal nucleus), the images were imported into ImageJ (1.37) and the percentage of area occupied by the staining was measured using the threshold parameter. Cells count was assed manually using ImageJ (1.37). Analysis was performed in double blinded to avoid bias of analysis, each microglial cell body in hypoglossal nucleus were counted. Fluorescent staining of images was performed using a Zeiss LSM800 confocal microscope supported by the Zen software (2.3 system). Confocal images were then processed using Fiji (ImageJ Version 2.0.0-rc-43/1.51n).

Statistical Analyses and Figure Preparation

Data are presented as mean ± standard error of the mean (SEM). Statistical analyses were carried with the Prism software (version 8.0, GraphPad Software Inc.). Values were considered statistically significant if $p < 0.05$. All panels were assembled using Adobe Photoshop CC 2018 (version 19.1.0) and Adobe Illustrator CC 2018 (version 23.0.1).

RESULTS

Mouse Model of CSF1R Deletion Specifically in Microglia

To delete CSF1R in microglia, we crossed CSF1R$^{fl/fl}$ mice with the CX3CR1-Cre^{ERT2} mice and exposed them to i.p. tamoxifen (TAM) injections as previously reported by us (6). After injection, Cre complex goes to the nucleus and interacts with the Lox site, which leads to excision of CSF1R gene Exon 5 (**Figure 1A**). In order to determine when the knock-out affect a maximum of cells, we have performed three different time-courses (**Supplementary Figure 1A**). The surgery made 13 days after the last tamoxifen injection provided the best results. Quantification of CSF1R in the hypoglossal nucleus shows a strong effect of the knock-out since the CSF1R expression was dramatically decreased (**Figures 1B,C**). To further test the relevance of our model, we used CSF1R-loxP-CX3CR1-cre/ERT2,Rosa^{tm14} mice. Mice express robust tdTomato fluorescence following Cre-mediated recombination and a large amount of CX3CR1-positive cells in the brain are affected by the knock-out (**Figure 1D**). Quantification shows that 82.9% of Iba-1$^+$ cells are Rosa^{tm14+} (**Figure 1E**). These data indicate that our model is reliable, and strongly efficient to delete CSFR1 selectively in microglia.

Microglial Proliferation in Hypoglossal Nucleus Is Maximal 7 Days After the Lesion

Hypoglossal nerve lesion causes a robust proliferation of microglia in the ipsilateral side of the nucleus, especially at time 7 days post injury (**Figures 2A,B**). Although few new cells are detected 24 h after the lesion, these are quite numerous at 4 and 7 days in the ipsilateral side of hypoglossal nerve-injured mice. After this time point, the number of new Iba1 positive cells slowly decreased to a basal level at day 31 after lesion (**Figure 2B**). Considering that the peak proliferating level is 7 days post-surgery, we selected this time point to determine the potential role of CSF1R in this mechanism.

Knocking-Out CSF1R Selectively in Microglia Does Not Affect Cell Proliferation

As previously described, a marked proliferation of microglia takes place in the ipsilateral hypoglossal nucleus 7 days after the systemic nerve injury. Following our protocol, CSF1R-loxP-CX3CR1-cre/ERT2 mice had surgery 13 days after last tamoxifen injection to allow the complete knock-out in microglia (**Figure 3G**). Taking into consideration that CSF1R signaling pathway is known to be essential for microglia proliferation in pathological conditions (14, 15), our data are unexpected. Indeed, CSFR1 deletion did not affect microgliosis and a strong proliferation of Iba-1$^+$ cells was observed in hypoglossal nucleus of CSF1R knock-out group (**Figure 3A**). There were actually no significant differences between WT and cKO groups (**Figures 3B,F**) and at all the time courses tested (**Supplementary Figure 1**). To further validate this surprising result, we determined the Iba-1 protein levels by Western blot and found a similar amount of Iba-1 levels in the brain of both groups of mice (**Figure 3C**). These results are quite interesting because they indicate that knocking-out CSF1R in microglial cells in a non-pathologic context does not impair their proliferation (**Figure 3F**). These findings put in light on the subjective importance of CSF1R signaling pathway depending on a pathologic or non-pathologic situation. We have also noticed that the morphology of microglia in the CSF1R cKO mice seemed different from those of control mice (**Figure 3E**). They have more ramifications and their cell bodies are thicker in CSF1R-deficient mice. This phenotype match with the shape observed in TREM2 overexpression cells (16) (**Figure 3D**) and this results could be the clue of a compensating mechanism overcoming the CSF1R deletion.

CSF1R Depletion Induces Infiltration of Peripheral Cells

These results lead us to wonder if cells from the periphery could compensate and replenish Iba1-positive cells in the ipsilateral hypoglossal nucleus in the CSFR1-deficient mice after TAM injection. We then generated chimeric mice using chemotherapy-based regimen (**Figure 4A**). This method was used because it does not impact the integrity of the blood-brain barrier, which is limiting the infiltration of bone marrow-derived cells (17). Once the chimerism was confirmed by flow-cytometry, we injected TAM and then 13 days later, mice underwent nerve injury as

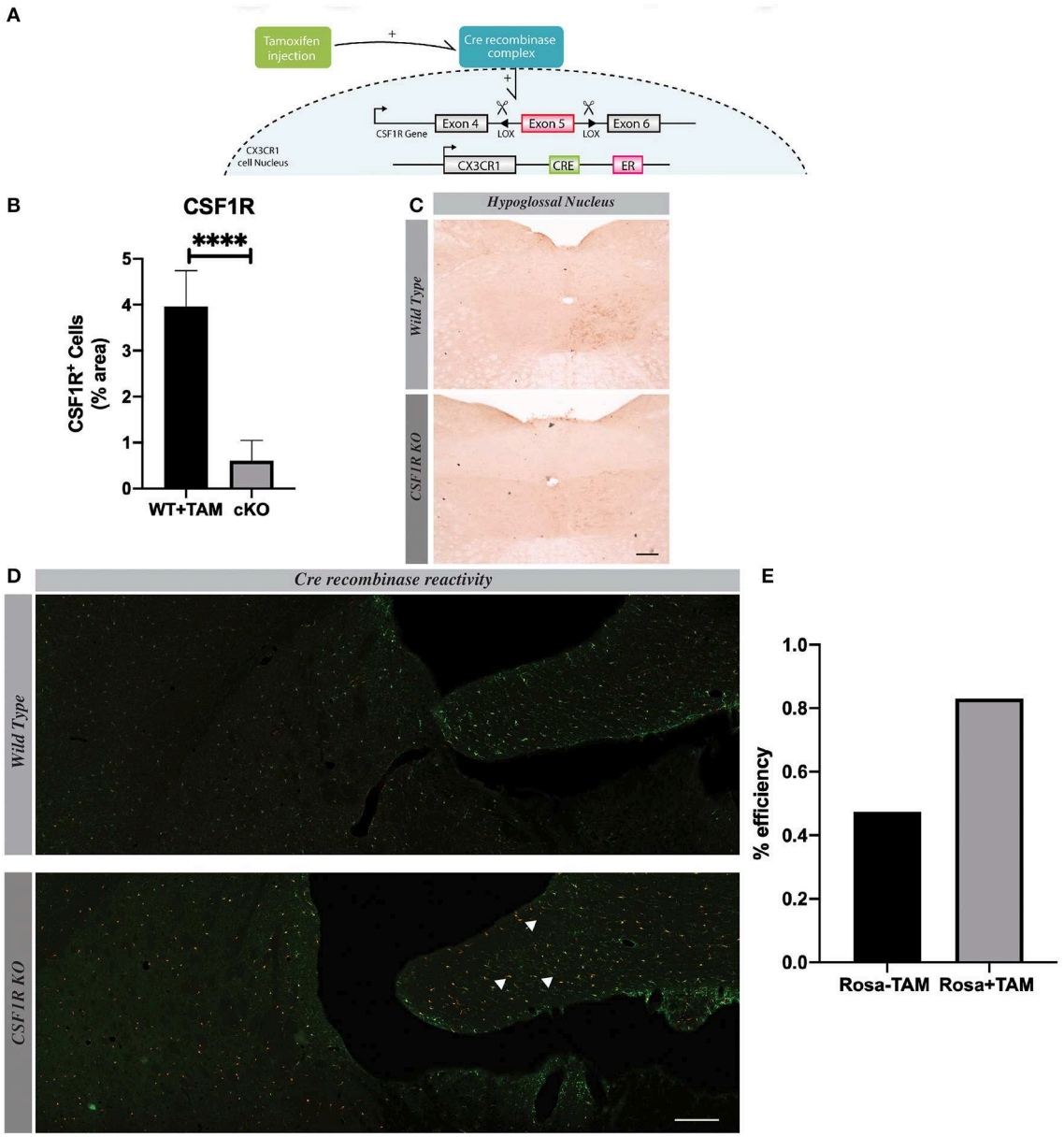

FIGURE 1 | CSF1R is deleted specifically in microglia. **(A)** We Show the genetic construction of CSF1R Ko mice. **(B)** Quantification presented as percentage of area occupied by staining, measured in the hypoglossal nucleus. Values are expressed as means ± SEM. Statistical analyses were performed using *t*-test $^{****}p < 0.001$ significantly different from cKO group. **(C)** Representative images of CSF1R staining in hypoglossal nucleus 7 days after lesion. **(D)** Confocal images showing co-localization of RedTomato positive cells (red) with Iba1 immunoreactive cells (green). **(E)** Quantification of RedTomato staining. Mice were injected with tamoxifen. White arrows point-out some examples of co-localization. $n = 7$ mice. Scale bar 200 μm.

described in the protocol above. We stained chimeric brains with antibody against Iba-1 to unravel infiltrating GFP-positive cells vs. resident microglia. Unlike wild type animals, numerous GFP cells were found in the brain CSF1R knock-out mice (**Figure 4B**). Bone marrow-derived cells infiltrated different regions, such as the area postrema and the hypoglossal nucleus. However, the number of Iba1$^+$ cells remained similar between wild type and knock-out mice despite the fact that 38.4% of GFP$^+$ cells were also Iba-1$^+$ in contrast to cuprizone model (**Figure 4C**). These data are quite interesting because bone marrow-derived cells are naturally attracted when CSF1R is deleted in resident microglia, but the role of these infiltrating cells are not yet well-understood (**Figure 4B**).

To better understand the role of infiltrating cells we have quantified the number of Tmem119 positive cells in hypoglossal nucleus area and Tmem119 gene expression, this marker is specific to microglia (18) (**Figures 4D,E**). There was no significative difference in Tmem119 cell count and gene expression between groups suggesting that most proliferating microglia (61.6%) derive from resident cells. Moreover, to

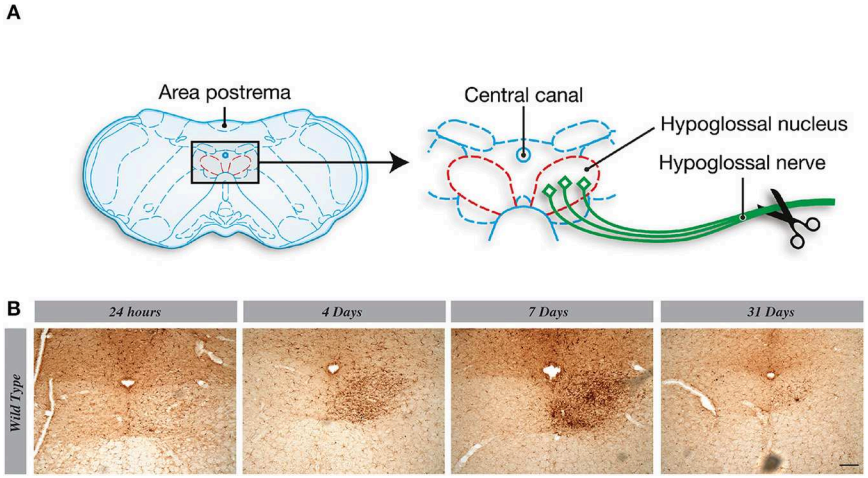

FIGURE 2 | Microglial proliferation in hypoglossal nucleus is maximal 7 days after the lesion. **(A)** Scheme of hypoglossal nerve transection surgery. **(B)** A progressive increase of Iba1 staining is observed from 24 h to 7 days post axotomy, which declined at 31 days. $n = 3$ mice. Scale bar: 100 μm.

complete the study we have verified whether bone marrow-derived cells were positive for Tmem119 and we did not find such positive GFP + Tmem119 cells confirming our findings (**Supplementary Figure 1C**). We have also determined the expression levels of different chemokines that could be involved in such infiltration process following TAM injection, but we did not see significant changes between WT and cKO brains (data not shown).

CSF1R-Depleted Microglia Affect Their Proliferation in the Cuprizone Diet Model of Acute Demyelination

Cuprizone-induced demyelination is an experimental mouse model to study different pathological events, especially in the progressive forms of MS. Myelin debris induce microglial activation and microgliosis via CSF1R, which plays a critical role in the clearance of myelin debris for a proper remyelination (6). Following a cuprizone diet for 5 weeks, we have deleted CSF1R selectively in microglia using CSF1R$^{fl/fl}$ CX3CR1-Cre^{ERT2} mice. When exposed to tamoxifen, these mice exhibited a significant reduced microgliosis in corpus callosum (**Figures 5A,E**). The quantification of Iba1^{+} cells shows a significant difference between WT and CSF1R$^{fl/fl}$ CX3CR1-Cre^{ERT2} groups of mice (**Figure 5B**). It is important to note that the number of Iba-1^{+} cells is no longer different between both groups after the remyelination process several weeks when cuprizone is removed from the diet indicating that CSF1R does not affect survival but proliferation of microglia (**Figures 5C,D**). These data underline a critical role of CSF1R for microgliosis in this mouse model of progressive MS.

DISCUSSION

Our results show that inhibition of the CSF1R pathway in microglia in a non-pathologic context does not impair microglial proliferation, which indicates that mCSF receptor is not necessarily implicated in proliferation in this model. CSF1R is known to play a major role in the proliferation and survival of several cell types in various models of diseases (19, 20). Curprizone model mimics the myelin loss observed in MS, especially the primary and secondary progressing form where proliferating microglia play a key role to remove myelin debris to allow a proper remyelination process when mice are no longer exposed to the toxin. In a previous study, CSF1R cKO animals exhibited a heavy myelin debris burden in the corpus callosum along with a reduced number of microglia when compared to their controls. The immune response associated with the remyelination process was also impaired (6).

On the other hand, the hypoglossal nerve lesion is a model to study the marked microglial proliferation in a non-brain pathological context after sectioning the nerve at the peripheral level. Our study aimed to study the role CSF1R pathway in such a phenomenon by deleting the gene using the conditional Cre/Lox system, which was highly efficient and effective as revealed with the CSF1R-loxP-CX3CR1-cre/ERT2, Rosa^{tm14} mice. However, our approaches failed to validate the potential role of the mCSF-CSFR1 pathway in microglial proliferation or survival following several time courses post hypoglossal nerve lesion. It suggests that CSF1R is not involved or is not the only receptor involved in these processes. This may not be explained by the low expression level of the Cre/Lox recombinase since most of Iba1-positive cells in hypoglossal nucleus were also red in the Rosa mice. These data suggest another mechanism underlying the survival and proliferation of resident microglia following a non-pathological brain condition.

Other studies have demonstrated a critical role of CSF1R in microglia survival by using a specific tyrosine kinase inhibitor in healthy mice (21). Using a molecule to shut down a signaling pathway is quite different from using a cell specific inducible gene deletion. The molecule may affect various populations of

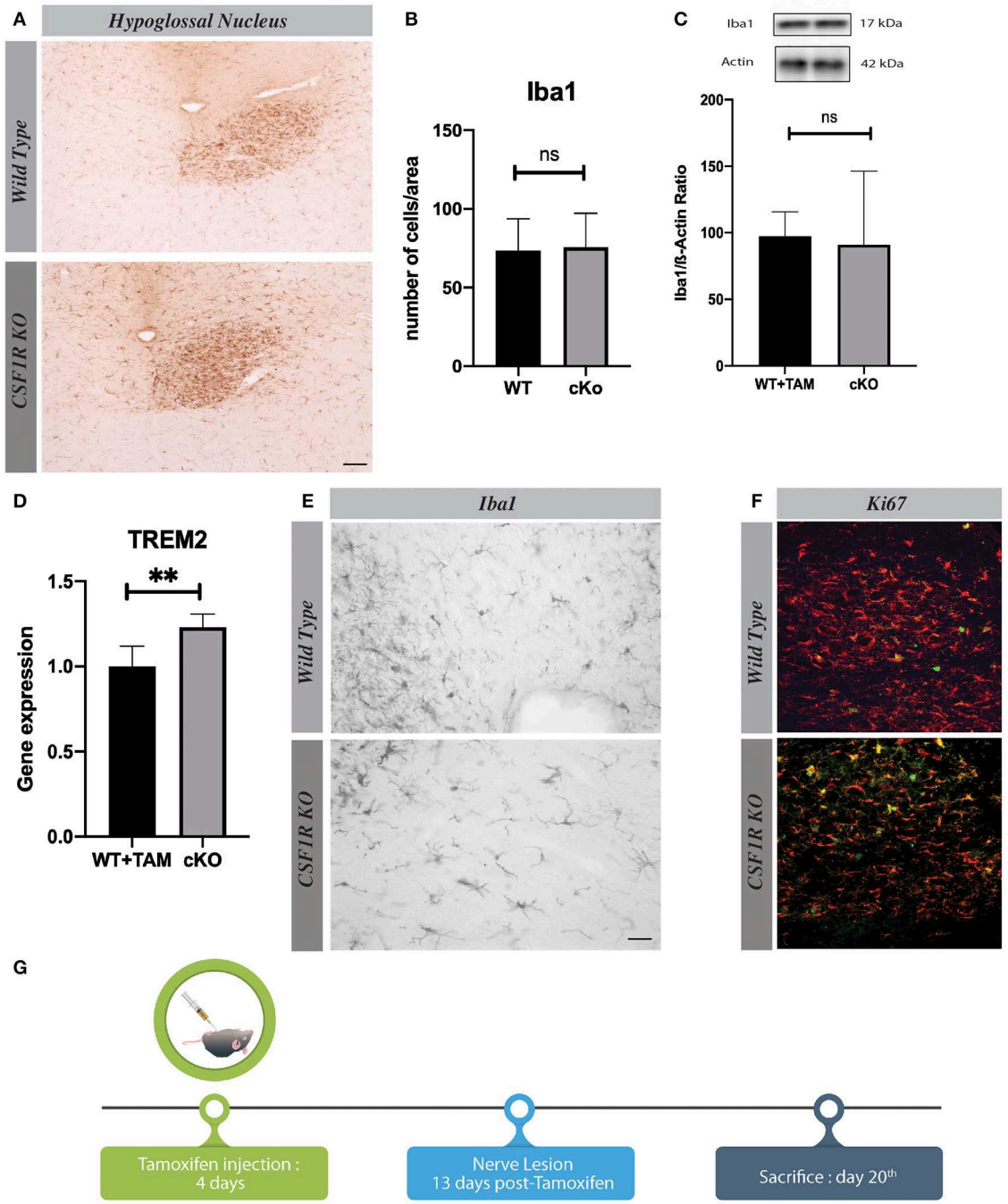

FIGURE 3 | Knocking-out CSF1R selectively in microglia does not impair microglial cell proliferation. **(A)** Representative images of Iba1 staining in hypoglossal nucleus, using different timelines, and **(B)** their quantification presented as percentage of area occupied by Iba1 staining measured in hypoglossal nucleus. **(C)** Level of Iba1 in cerebellum by Western blot. **(D)** Quantification of TREM2 gene expression in cerebellum by RT-Q-PCR. **(E)** Representative images of microglial morphology in cKO and WT mice. **(F)** Representative images of Iba1 (red) and Ki67 (green) staining in hypoglossal nucleus area. **(G)** Time course used for analyses. Values are expressed as means ± SEM. Statistical analyses were performed using *t*-test, $p < 0.005$. Scale bar 100 μm. $n = 3$–5 mice. $^{**}p < 0.01$.

cells and not specifically microglia, which may explain these different outcomes. Here the Cre/Lox system is restricted to CX3CR1 cells that does not affect their proliferation after hypoglossal nerve lesion but seems to change their phenotypes. Indeed, the structure of CSF1R knock-out microglia are different with their cell bodies that are sharper and darker, and they exhibit more ramifications. Triggering receptor expressed on myeloid cells 2 (TREM2) pathway could be involved in these structural changes, which have been reported in presence of TREM2 overexpression (22). This receptor is also known to

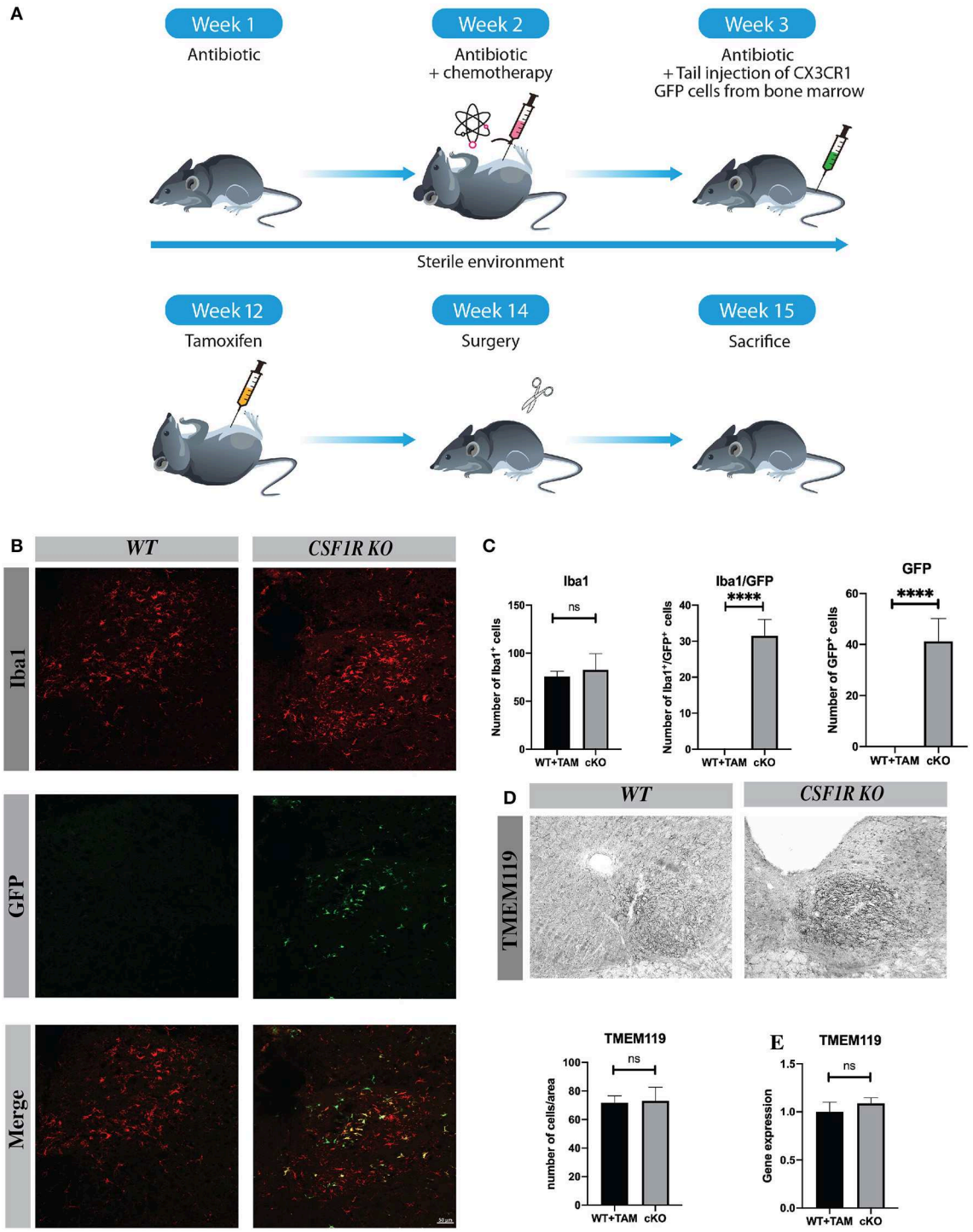

FIGURE 4 | CSF1R depletion induces infiltration of peripheral cells. **(A)** Scheme of the protocol to initiate chimeric mice. **(B)** Images of hypoglossal nucleus showing, infiltrating cells (green) and Iba1 positive cells (red), and infiltrating cells/Iba1 (yellow). Scale bar: **(A)** 50 μm. **(C)** The number of Iba1$^+$, GFP$^+$, and Iba1$^+$/ GFP$^+$ was counted in hypoglossal nucleus. **(D)** Tmem119 staining and cells count in hypoglossal nucleus area **(E)** Quantification of Tmem119 gene expression in cerebellum by RT-Q-PCR. Values are expressed as mean ± SEM. Statistical analyses were performed using *t*-test ****$p < 0.001$ significantly different from cKO group. $n = 3$–5 mice.

take part in microglia activation and survival (16) and there is a link between CSF1R and TREM2 with DAP12. DAP12 is a mediator of CSF1R proliferation pathway through MAPK and Akt. DAP12 ITAM domain is phosphorylated following CSF1R activation triggering ß catenin, a molecule that acts on cellular cycle (1). TREM2/DAP12- mediated signal also promotes

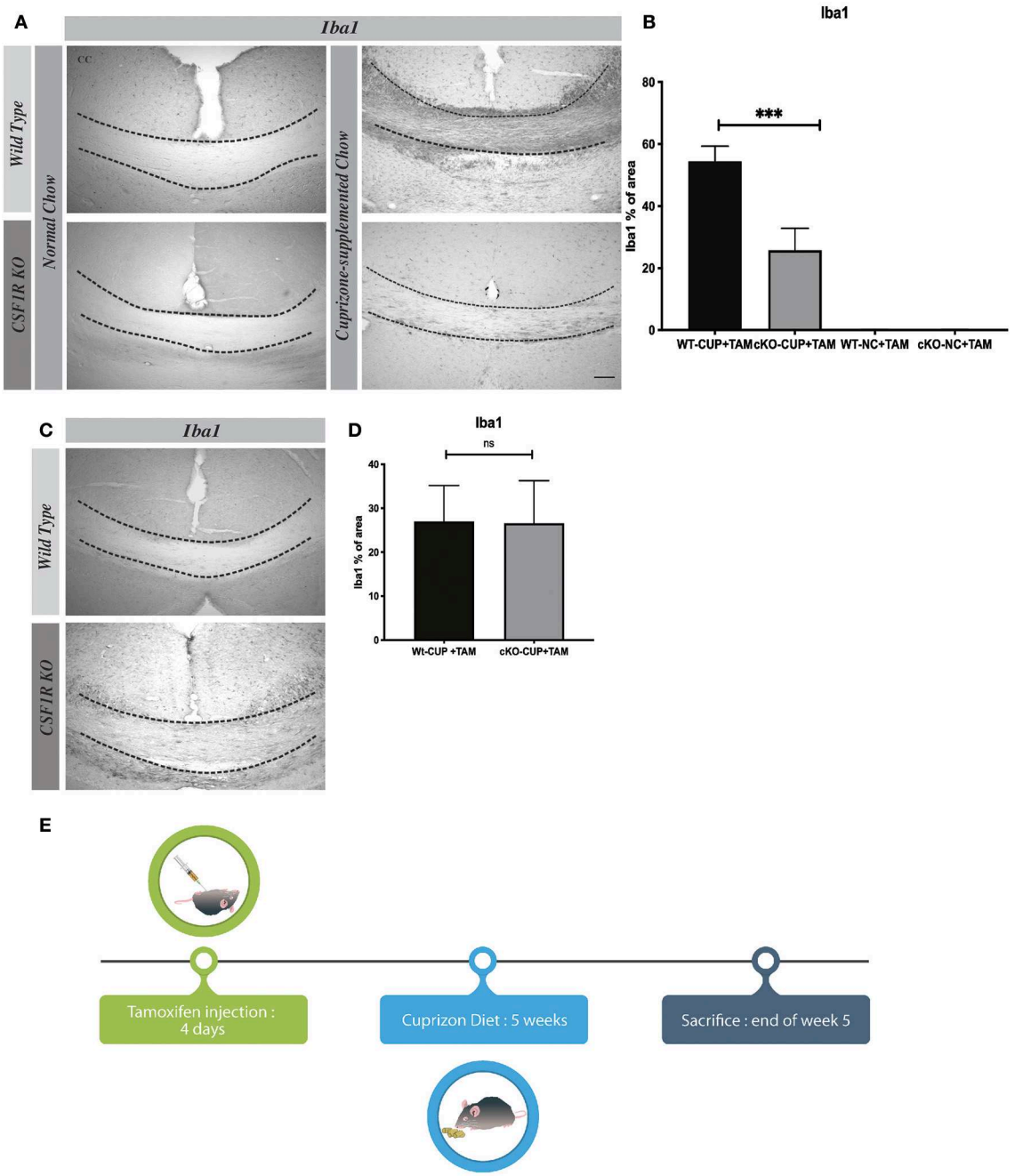

FIGURE 5 | In Cuprizone Diet model CSF1R depleted-microglia affect their proliferation. **(A)** Representative images of Iba1 staining in corpus callosum and **(B)** their quantification presented as percentage of area occupied by the staining, measured in the corpus callosum. Cuprizone supplemented chow (CUP) or Normal Chow (NC). **(C)** Representative images of Iba1 staining in corpus callosum and **(D)** their quantification presented as percentage of area occupied by the staining, measured in the corpus callosum, 5 days after cuprizone withdraw. **(E)** Timeline cuprizone protocol. Value are expressed as means ± SEM. Statistical analyses were performed using *t*-test $^{***}p < 0.001$ significantly different from group cKO-CUP + TAM. $N = 3$–6 mice. Scale bar: **(A)** 100 μm.

proliferation of microglia (22). In this regard, we have observed a significant increased expression of TREM2 in the brain of CSF1R-deficient mice (**Figure 3D**). The ablation of CSF1R could lead to a compensation by the overexpression of TREM2 and DAP12 phosphorylation in microglia explaining their phenotypic changes without affecting their proliferation.

Interestingly, CSF1R suppression leads to infiltration of circulating CX3CR1-positive cells after the surgery, although most of the proliferating microglia were resident Iba1 cells in hypoglossal nucleus. This was confirmed by the count and the expression levels of transmembrane protein 119 (Tmem 119) mRNA expression, a specific marker of microglia

(18) that remained the same between WT and cKO groups (**Figures 4D,E**). The presence of bone marrow-derived cells is not yet well-understood, as well as the method of recruitment. The chemotherapy-based regiment used to make chimeric mice does not affect the blood-brain barrier (17) and there is no such recruitment in wild-type mice after hypoglossal nerve lesion at any of the time evaluated.

Our study aimed to understand the role of CSF1R in microglial proliferation and survival in a non-pathologic context. We have used two different models, one mimicking the demyelination in progressive MS and in another one of pure and sterile proliferative microglia not associated with pathological conditions in the brain parenchyma. On one hand CSF1R-deleted mice feed with cuprizone exhibited an important drop of microglial cells in corpus callosum area, indicating the vital role of CSF1R signaling pathway for microglia proliferation in this context. On the other hand, CSF1R knock-out mice exhibited a marked microgliosis with no sign of impairment in response to a systemic nerve section. These data are quite novel since most studies that provided solid evidence for the essential role in the mCSF-CSF1R pathway in microgliosis were actually during various pathologies of the CNS. The mechanisms mediating these effects after hypoglossal nerve lesion have yet to be unraveled and whether TREM2 overexpression compensates for CSF1R deletion in microglia will be investigated in future studies.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by CPAUL3, Laval University.

AUTHOR CONTRIBUTIONS

VP participated in the design of the experiments, analyzed and interpreted the data, wrote the manuscript, and assembled the figures. NL and PP participated in the design of the experiments, analyzed and interpreted the data, and assembled the figures. SR formulated the study concept and all experimental designs, supervised the project, and wrote and revised the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.00047/full#supplementary-material

Supplementary Figure 1 | Timelines. **(A)** Different timelines used for the hypoglossal nerve transection study. **(B)** Timeline used for the cuprizone model. **(C)** Image of hypoglossal nucleus (left) and parenchyma (right) in chimeric mice showing infiltrating cells (green) and Tmem119 (red). Scale bar: 100 μm.

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ORIGINAL RESEARCH
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Siponimod (BAF312) Activates Nrf2 While Hampering NFκB in Human Astrocytes, and Protects From Astrocyte-Induced Neurodegeneration

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Multiple sclerosis (MS) is an inflammatory neurodegenerative disease of the central nervous system (CNS) with heterogeneous pathophysiology. In its progressive course oligodendrocyte and neuroaxonal damage is sustained by compartmentalized inflammation due to glial dysregulation. Siponimod (BAF312), a modulator of two sphingosine-1-phosphate (S1P) receptors (S1P1 and S1P5) is the first oral treatment specifically approved for active secondary progressive MS. To address potential direct effects of BAF312 on glial function and glia-neuron interaction, we set up a series of *in vitro* functional assays with astrocytes generated from human fibroblasts. These cells displayed the typical morphology and markers of astroglia, and were susceptible to the action of inflammatory mediators and BAF312, because expressing receptors for IL1, IL17, and S1P (namely S1P1 and S1P3). Targeting of S1P signaling by BAF312 inhibited NFκB translocation evoked by inflammatory cytokines, indicating a direct anti-inflammatory activity of the drug on the human astrocyte. Further, while glia cells exposed to IL1 or IL17 downregulated protein expression of glutamate transporters, BAF312-treated astrocytes maintained high levels of GLAST and GLT1 regardless of the presence of inflammatory mediators. Interestingly, despite potential glial susceptibility to S1P signaling via S1P3, which is not targeted by BAF312, NFκB translocation and downregulation of glutamate transporters in response to S1P were inhibited at similar levels by BAF312 and FTY720, another S1P signaling modulator targeting also S1P3. Accordingly, specific inhibition of S1P1 via NIBR-0213 blocked S1P-evoked NFκB translocation, demonstrating that modulation of S1P1 is sufficient to dampen signaling via other S1P receptors. Considering that NFκB-dependent responses are regulated by Nrf2, we measured activation of this critical transcription factor for anti-oxidant reactions, and observed that BAF312 rapidly induced nuclear translocation of Nrf2, but this effect was attenuated in the presence of an inflammatory milieu. Finally, *in vitro* experiments with spinal neurons exposed to astrocyte-conditioned media showed that modulation of

S1P or cytokine signaling in astrocytes via BAF312 prevented neurons from astrocyte-induced degeneration. Overall, these experiments on human astrocytes suggest that during neuroinflammation targeting of S1P1 via BAF312 may modulate key astrocyte functions and thereby attain neuroprotection indirectly.

Keywords: astrocytes, BAF312, fingolimod, neurodegeneration, neuroinflammation, NFκB, Nrf2, siponimod

INTRODUCTION

Multiple sclerosis (MS) is a complex, highly debilitating inflammatory disease of the central nervous system (CNS) and represents the most common cause of neurological disability in young adults (1). Most of the available drugs display efficacy in the relapsing-remitting (RR-MS) form of the disease (2), where frequent waves of infiltrating immune cells into the CNS lead to demyelination, but not in progressive MS, where oligodendrocyte and neuroaxonal damage is sustained by compartmentalized inflammation due to glial dysregulation (3). After several years of disease most of the RR-MS patients enter the progressive stage (called secondary progressive MS, SP-MS), characterized by steady accumulation of disability in absence of acute clinical events (4). Sphingosine 1-phosphate (S1P)–S1P receptor axis is a known pharmacological target in MS, due to its key role in the regulation of immune cell migration from peripheral lymphoid organs to CNS (5). S1P signals through five G protein-coupled receptors (S1P1-5), which are widely expressed and control several cellular processes, such as growth, survival and differentiation (5). Neurons and glia cells in the CNS may bear S1P receptors (6, 7), opening to the possibility of interfering with events occurring in the nervous tissue via targeting S1P signaling pathway. Fingolimod (FTY720), the first oral therapy approved for RR-MS, is a prodrug that, after activation by phosphorylation, binds to all S1P receptors with the exception of S1P2 (8), and thereby induces lymphopenia (9), reduces the inflammatory activation of circulating and CNS-resident myeloid cells (10–13), and blocks astrocyte activation during neuroinflammation (7, 14, 15). Despite the lack of efficacy for fingolimod in progressive MS (16), the potential neuroprotective effects due to the blockade of S1P-S1P receptor axis in CNS prompted the development of novel S1P receptor modulators which work as active drugs. The recent phase-3 EXPAND trial demonstrated that oral administration of siponimod (BAF312), which targets S1P1 and S1P5 (17), attenuates the risk of disability progression in SP-MS, with a major effect in those patients with inflammatory disease (18). For this reason the European Medicines Agency recommended BAF312 as first oral treatment for active SP-MS in November 2019[1]. BAF312 treatment significantly hinders lesion enlargement and brain atrophy after 12 months (18), demonstrating relevant protective properties in CNS tissue via mechanisms which remain to be clarified. *In vitro* models for human astrocytes can be generated from readily accessible cells, such as fibroblasts, and provide the unprecedented possibility to explore the contribution of this glia cell population to human diseases, study its interaction with neuronal cells and test potential neuroprotective drugs. To address direct effects of BAF312 on glial function and glia-neuron interaction, we generated human astrocytes from reprogrammed fibroblasts and set up a series of *in vitro* assays to verify whether BAF312 may hamper glial inflammatory activity and support physiological and anti-oxidant functions of the astrocyte.

[1]https://www.ema.europa.eu/en/medicines/human/summaries-opinion/mayzent

MATERIALS AND METHODS

Fibroblast Reprogramming and Differentiation Into iAstrocytes

Human skin biopsies were obtained from two healthy subjects after signing of informed consent approved by the Ethics Committee of Ospedale San Raffaele. Fibroblasts were isolated and reprogrammed to generate human iPSC clones with the Sendai virus technology (CytoTune-iPS Sendai Reprogramming Kit, Thermo Fisher Scientific) (19). iPSC clone characterization is described in (20). Human neural precursor cells (hiPSC-NPCs) were generated with the dual SMAD inhibition (SB431542/Dorsomorphin)/Hedgehog pathway activation (SAG/Purmorphamine)/WNT pathway activator (CHIR99021) and maintained in proliferation medium as described in (21). For astrocyte differentiation, the iNPCs were seeded at low density in Geltrex (Thermo Fisher Scientific)-coated T75 flasks (2×10^6 cells/flask) for 24 h. The day after, proliferation medium was changed to DMEM supplemented with 1% antibiotics, 200 mM L-Glutamine, 100 mM Sodium Pyruvate (Thermo Fisher Scientific), 10% FCS and 0.3% N2 (22). Astrocytes were allowed to differentiate for several weeks, detached using trypsin and checked for morphology and marker expression at different time points. Phase contrast images for morphologic assessment were obtained at Leica DMIL LED microscope.

Stimulation of Human iAstrocytes

Human iAstrocytes were incubated with 100 nM Fingolimod (FTY720-phosphate, Selleckchem), 100 nM Siponimod (BAF312, Selleckchem) or 1 μM NIBR-0213 (Merck) or vehicle (PBS or DMSO max 0.4% v/v) for 1 hour. Cells were then treated with IL1β (10 ng/ml, Thermo Fisher Scientific), IL17 (10 ng/ml, Peprotech) or S1P (100 nM, Echelon Biosciences). Incubation times were 1 h for S1P1 internalization assay, 30 min for NFκB assay, 1, 2 or 4 h for Nrf2 assay or 24 h for glutamate transporter assay. Cells were then processed for immunofluorescence and stained with appropriate primary antibodies. For the generation of astrocyte conditioned media iAstrocytes were pre-incubated with drugs, and then exposed to the inflammatory stimuli for 8 h. Astrocyte medium was replaced with fresh neuronal medium and, after additional 24 h culture, supernatants were collected,

centrifuged to remove cell debris, and stored at −80°C. Before addition to primary neurons, astrocyte supernatants were diluted down to 1:4 with medium.

RNA Extraction, cDNA Synthesis, and Qualitative PCR

Total RNA was extracted by Tri Reagent Solution (Thermo Fisher Scientific) and reverse transcribed using random hexamer primers and Superscript III reverse transcriptase (all from Thermo Fisher Scientific) following the manufacturers' instructions. To remove contaminating DNA, RNA was treated with DNaseI enzyme (Thermo Fisher Scientific). As positive control, human peripheral blood mononuclear cells (PBMC) were isolated from a healthy donor as described in (10) and total RNA was extracted. Qualitative RT-PCR was performed using GoTaq G2 DNA polymerase (Promega) and dNTPs set (Thermo Fisher Scientific). The sequences of used primers are as follows: 5′-GGA GTA GTT CCC GAA GGA CC-3′ (sense) and 5′-TCT AGA ATC CAC GGG GTC TG-3′ (antisense) for S1P5 receptor (236-bp product), 5′-GAT GAC ATC AAG AAG GTG GTG AA-3′ (sense) and 5′-GTC TTA CTC CTT GGA GGC CAT GT-3′ (antisense) for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (246-bp product). Thirty-two cycles of amplification were performed at 94°C for 30 s, at 60°C for 30 s, and at 72°C for 1 min. PCR products were separated by electrophoresis on 2% agarose gel and visualized with SYBR Safe (Thermo Fisher Scientific) staining.

Generation and Treatment of Primary Spinal Neurons

Primary spinal neurons were obtained from 16-day-old Sprague Dawley rat embryos as described (7, 23). Briefly, embryonal spinal cords, depleted of spinal root ganglia, were dissected, carefully minced and digested for 15 min at 37°C with 500 μg/ml DNAsi I (Roche) and 0.25% trypsin (Thermo Fisher Scientific) in L-15 medium (Thermo Fisher Scientific) supplemented with antibiotics. After digestion, tissue homogenate was washed 3 times with L-15 medium and finally cultured in Neurobasal medium (Thermo Fisher Scientific) supplemented with 10 ng/ml glial cell-derived neurotrophic factor (GDNF; Sigma), 20 ng/ml fibroblast growth factor (FGF; Peprotech), 50 μg/ml insulin (Sigma), B27 supplement (Thermo Fisher Scientific), 1% FCS (Euroclone), and 10 mM Glucose. Cells were seeded on poly-D-lysine and collagen (both from Sigma) coated glass coverslips. After 24 h 15 μM Cytosine b-D-arabinofurnoside (AraC; Sigma) was added to cultures and left for 4 days to eliminate contaminating microglia cells, astrocytes and oligodendrocytes. Neurons were exposed to astrocyte conditioned media for 8 h, then processed for immunofluorescence and stained with monoclonal antibody against β-tubulin. All nuclei were stained with DAPI. For assessment of neuronal counts, the numbers of DAPI positive nuclei were quantified and reported as percentage of control (neurons exposed to supernatants from vehicle-treated astrocytes; sCTRL). Neuronal network was measured by β-tubulin signal and expressed as percentage of controls.

Immunofluorescence Experiments

Astrocytes or neurons were plated on coverslips, fixed in 4% PFA or MetOH, permeabilized with 0.2% Triton X-100 (Merck), blocked in PBS + 1% BSA (Merck) + 5% FCS and stained with primary antibodies. Then, cells were incubated with appropriate species-specific Alexa Fluor 488/594-conjugated secondary antibodies (Thermo Fisher Scientific), counterstained with 4′,6-diamidino-2-phenylindole (DAPI, Sigma) and mounted with fluorescent mounting medium (Agilent). The following primary antibodies were used: rabbit anti-GFAP (Agilent), mouse anti-nestin (Merck Millipore), mouse anti-vimentin (Abcam), rabbit anti-S100β (Abcam), rabbit anti-EDG1 (Santa Cruz Biotechnology), rabbit anti-EDG3 (Santa Cruz Biotechnology), mouse anti-IL1R (R&D), rabbit anti-IL17R (Santa Cruz biotechnology), rabbit anti- NFκB p65 (Abcam), rabbit anti-GLAST (Abcam), guinea pig anti-GLT1 (Merck Millipore), rabbit anti-Nrf2 (Abcam), mouse anti Neuronal Class III β-Tubulin (Covance). The following secondary antibodies were used: Alexa Fluor 488 donkey anti-rabbit IgG (H + L), Alexa Fluor 594 donkey anti-rabbit IgG (H + L), Alexa Fluor 488 donkey anti-mouse IgG (H + L), Alexa Fluor 594 donkey anti-mouse IgG (H + L), Alexa Fluor 488 goat anti-guinea pig IgG (H + L) (all from Thermo Fisher Scientific). Fluorescence images were captured at fluorescence microscope (Leica DM5500B) or Leica TCS SP5 confocal laser-scanning microscope equipped with 40× oil objective. LASAF and LASX softwares were used for image acquisition, and ImageJ (download at: http://rsbweb.nih.gov/ij/) software was used for image analysis. To quantify nuclear NFkB and Nrf2, DAPI images were converted to 8-bit, and regions of interest (ROIs) were generated to select (DAPI positive) nuclei. Then ROIs were applied to the corresponding NFkB or Nrf2 images, fluorescence thresholds were fixed on the unstimulated condition, and the fraction of positive nuclei was assessed (an example of analytical strategy is depicted for Nrf2 in **Supplementary Figure S3**). Similarly, the fraction of highly fluorescent astrocytes above the threshold was used to quantify cellular GLAST and GLT1 expression under the distinct conditions.

Statistical Analyses

Data in figures are presented as mean ± standard deviation (SD) or standard error of the mean (SEM) as indicated in figure legends. The exact number of independent experiments performed is reported in figure legends. Unpaired *t*-test was performed to compare means. All *p*-values were two-sided and subjected to a significance level of 0.05. In figures, asterisks denote statistical significance as $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. Statistical analyses were performed in Excel or GraphPad Prism.

RESULTS

Generation and Characterization of Human iAstrocytes

Human fibroblasts were isolated from skin biopsies and reprogrammed to generate human iPSC clones, which were

differentiated first in iPSC-derived NPCs and then into mature astrocytes (hereon called iAstrocytes). Cells were cultured for several weeks and sampled at different time points to assess their morphology and phenotype. First, we observed a significant change in cell size and morphology during the differentiation process from iNPC, as human iAstrocytes clearly showed an increment in cell size and acquired the typical morphology of astroglia (**Figure 1A**). Immunofluorescence experiments confirmed that classical astrocyte markers were highly expressed in all analyzed cultures. In fact, iAstrocytes were positive for GFAP, S100ß, nestin and vimentin that remained expressed at high levels also at advanced culture stages (**Figures 1B,D**). As reactive astrocytes in MS lesions display coordinated upregulation of receptors for inflammatory cytokines (IL1R, IL17R) and the lipid mediator S1P (S1P1 and S1P3) (7), we checked protein expression of these receptors on human iAstrocytes and observed strong positivity for all of them (**Figures 1C,E**), indicating that our *in vitro* human cell model mimics the phenotype of the reactive glia cell in the human pathological tissue and may be responsive to inflammatory mediators. Human iAstrocytes did not express transcripts for S1P5, another possible target of BAF312 or FTY720 (**Supplementary Figure S1**).

BAF312 Blocks Inflammatory Activation of iAstrocytes and Supports Maintenance of Glutamate Transporters

We set up *in vitro* assays with our human cell system to study the effects of BAF312 and FTY720 on different astrocyte functions.

As these drugs were shown to induce rapid S1P1 internalization in rodent astrocytes (24, 25), we checked this phenomenon in our cells and confirmed that, differently from control cells, iAstrocytes displayed intracellular S1P1 aggregates with perinuclear distribution when exposed to FTY720 or BAF312 for 1 h (**Supplementary Figure S2**).

NFκB is a key transcription factor in cytokine and S1P signaling, and plays a pivotal role in the amplification of inflammatory and neurodegenerative processes (5, 26). We verified whether our human iAstrocytes activated NFκB in response to inflammatory cues and studied the effect of S1P signaling modulators on astrocyte behavior. As shown in **Figures 2A,B**, IL1 or IL17 strongly induced nuclear translocation of NFκB-p65 in human iAstrocytes, however, this effect was blocked by astrocyte exposure to BAF312 or FTY720.

Maintenance of extracellular glutamate concentrations below neurotoxic levels is a critical function of glial glutamate transporters GLAST and GLT1 (27). Both transporters were expressed on resting iAstrocytes (CTRL; **Figures 2C,E**), but strongly downregulated in cells exposed to inflammatory cytokines for 24 h (**Figures 2C–F**). Differently, cells treated with BAF312 or FTY720 maintained high GLAST and GLT1 expression even under inflammatory conditions (**Figures 2C–F**).

Astrocytes may react to the mediator S1P via S1P1 and S1P3. While FTY720 targets both receptors, BAF312 is selective for S1P1 only, leaving open the possibility of responding to S1P via S1P3. To check this hypothesis we used the NFκB assay to measure astrocyte activation in response to S1P and

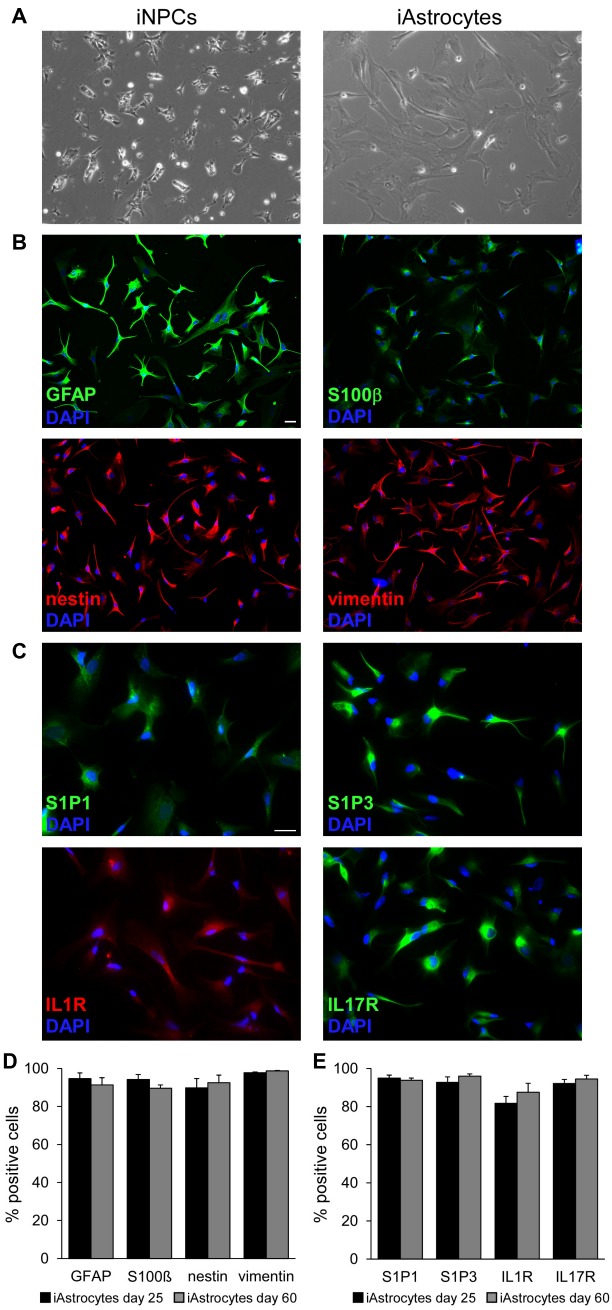

FIGURE 1 | Characterization of iPSC-derived astrocytes. **(A)** Phase contrast images showing human iNPC (left panel) and human iAstrocytes (right panel). **(B,C)** Representative immunofluorescence stainings for GFAP, S100β, nestin, vimentin **(B)** and S1P1, S1P3, IL1R and IL17R **(C)** in human iAstrocytes. DAPI was used for nuclear staining. **(D,E)** Percentage of cells positive for astrocyte markers at two timepoints during differentiation. Reported quantifications were performed on three different human iAstrocytes preparations from the same iNPC cell line. Bars represent SEM. Same observations were recorded in human iAstrocytes from a second iNPC cell line. Scale bar = 30 μm.

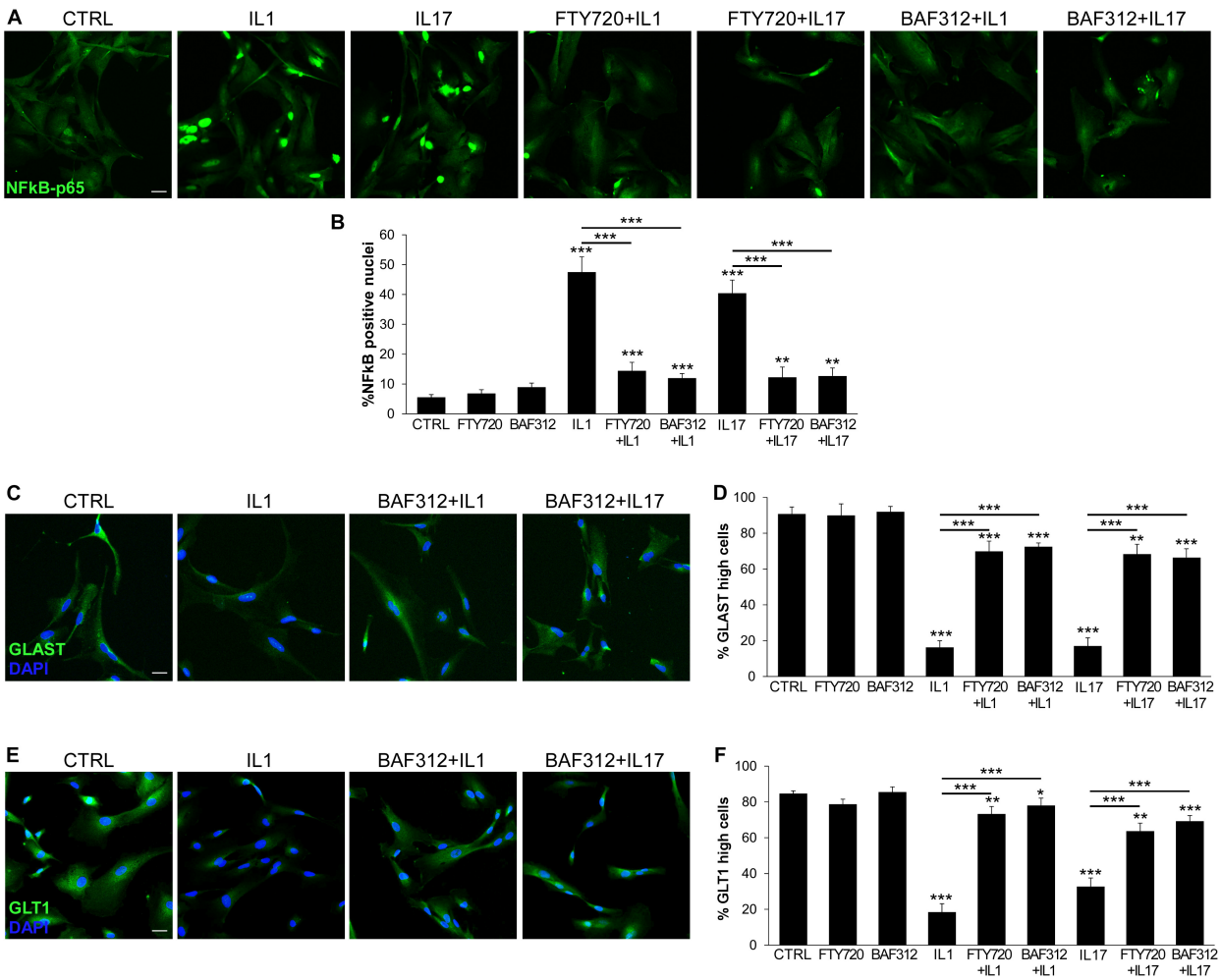

FIGURE 2 | BAF312 inhibits NFκB nuclear translocation and maintains glutamate transporters expression in astrocytes exposed to inflammatory cytokines. **(A)** Representative immunofluorescence stainings for NFκB-p65 in human iAstrocytes stimulated with cytokines alone or after pre-incubation with BAF312. **(B)** Graph reports the percentage of NFκB-p65 positive nuclei in human iAstrocytes under distinct conditions. **(C–F)** Representative immunofluorescence stainings for GLAST **(C)** and GLT1 **(E)** in human iAstrocytes and relative quantifications **(D,F)** under distinct conditions. Representative images are shown. DAPI was used for nuclear staining. Data are shown as mean ± SD of a representative experiment out of three independent experiments. Scale bars: 30 μm. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

differentially interfered with S1P signaling via FTY720 or BAF312. As shown in **Figure 3**, selective blockade of S1P1 and not S1P3 with BAF312 achieved similar, strong inhibition of S1P-induced NFκB-p65 translocation to that exerted by FTY720 (**Figures 3A,B**). As additional control, iAstrocytes were treated with NIBR-0213, a potent and selective S1P1 antagonist (28), and S1P-mediated NFκB-p65 nuclear translocation was assessed. Similarly to BAF312, specific inhibition of S1P1 via NIBR-0213 abolished S1P-evoked NFκB-p65 translocation (**Figures 3C,D**). S1P also downregulated protein expression of glutamate transporters on iAstrocytes (**Figures 3E–G**), however, this process was equally hindered by FTY720 and BAF312 (**Figures 3E–G**).

All together these data indicate that triggering of inflammatory signaling cascades in glia cells may be prevented by S1P receptor modulators, and that S1P1 targeting via BAF312 is sufficient to directly dampen inflammatory activation via other S1P receptors and support physiological astrocyte functions.

BAF312 Induces Nrf2 Activation in Human iAstrocytes

Activation of the transcription factor Nrf2 represents a key checkpoint for cellular antioxidant responses and its induction in astrocytes may confer neuroprotection during neuroinflammation (29). To ascertain whether BAF312 and FTY720 may regulate Nrf2 activation in glial cells, human iAstrocytes were stimulated with the drugs over a few hours and assessed for Nrf2 expression by immunofluorescence. Under resting conditions, iAstrocytes displayed mainly cytoplasmic Nrf2 expression (**Figure 4A**), however, exposure to S1P modulators for 1 h significantly increased Nrf2 nuclear levels

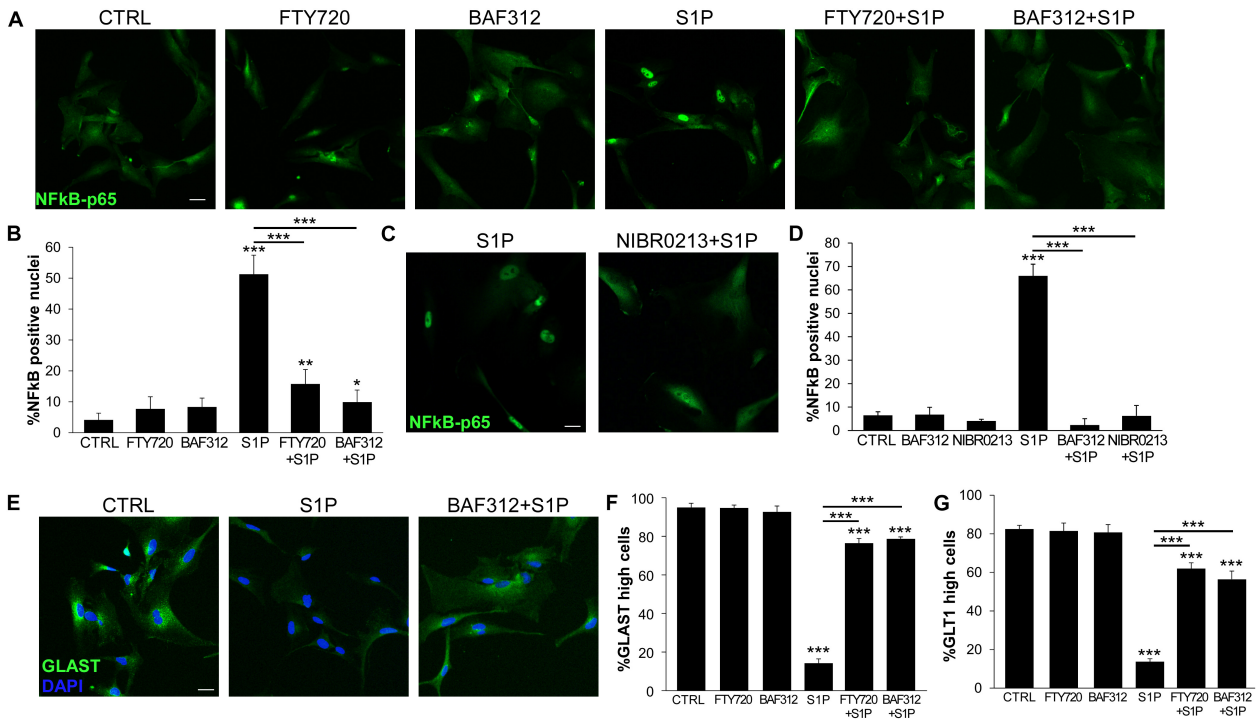

FIGURE 3 | S1P1 targeting is sufficient to support physiological astrocyte functions. **(A)** Representative immunofluorescence stainings for NFκB-p65 in human iAstrocytes stimulated with S1P alone or after pre-incubation with FTY720 or BAF312 and relative quantifications **(B)**. **(C)** Fluorescence images for NFκB-p65 in human iAstrocyte cultures stimulated with S1P eventually in the presence of NIBR-0213. Percentage of NFκB-p65 positive nuclei is reported in **(D)**. **(E)** Representative immunofluorescence stainings for GLAST in human iAstrocytes exposed to vehicle (CTRL), S1P alone or after pre-incubation with BAF312. **(F,G)** Frequency of GLAST **(F)** or GLT1 **(G)** high positive cells under the different experimental conditions. DAPI was used for nuclear staining. Data are shown as mean ± SD of a representative experiment out of three independent experiments. Scale bars: 30 μm. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

(**Figures 4A,B**), an effect that persisted over time (**Figure 4B**). We then checked whether this action was maintained under inflammatory conditions, and noted that Nrf2 induction was significantly reduced in the presence of IL1, IL17 or S1P (**Figures 4C–F**). These experiments indicate that S1P receptor modulators may directly activate protective responses in glial cells via Nrf2, but that their efficacy may be affected upon neuroinflammation.

BAF312 Hampers Astrocyte-Induced Neurodegeneration

To test the overall impact of astrocyte mediators on neurons, we exposed human iAstrocytes to the drugs and then to the inflammatory mediators for 8 h, changed the medium to remove stimuli and collected the supernatants after a further 24 h culture. Astrocyte-conditioned media were then added to pure cultures of spinal neurons, which were then assessed for cell number and morphology via DAPI and β-tubulin stainings. While supernatants from non-treated (sCTRL) or FTY720- or BAF312-treated cultures (sFTY720, sBAF312) did not affect neuronal survival and network integrity, conditioned media from human iAstrocytes stimulated with the inflammatory factors (sIL1, sIL17, and sS1P) triggered robust degenerative responses characterized by neuronal loss and neurite fragmentation (**Figures 5A–C**). However, when astrocyte media were generated in the presence of BAF312 or FTY720, their addition to spinal neurons did not trigger neurodegeneration despite astrocyte exposure to the inflammatory mediators (**Figures 5A–C**), indicating that astrocyte targeting by S1P receptor modulators may rescue neurons from astrocyte-induced degeneration.

DISCUSSION

In this study, we provided *in vitro* evidence of neuroprotective effects of BAF312 via the human astrocyte during inflammation. In particular, we (i) generated human fibroblast-derived astrocytes to measure pharmacological effects of S1P receptor modulators; (ii) addressed the direct impact of BAF312 on NFkB activation and glutamate transporters in astrocytes; (iii) demonstrated the direct activation of Nrf2 by S1P receptor modulators; and (iv) performed side-by-side comparisons between BAF312 and FTY720 in all *in vitro* assays. Importantly, BAF312 action on the astrocyte was strong enough to inhibit neurodegeneration triggered by glial mediators generated during neuroinflammation.

The recent results about the efficacy of the S1P receptor modulator BAF312 in the treatment of secondary progressive MS indicate that the slowdown of disability progression is

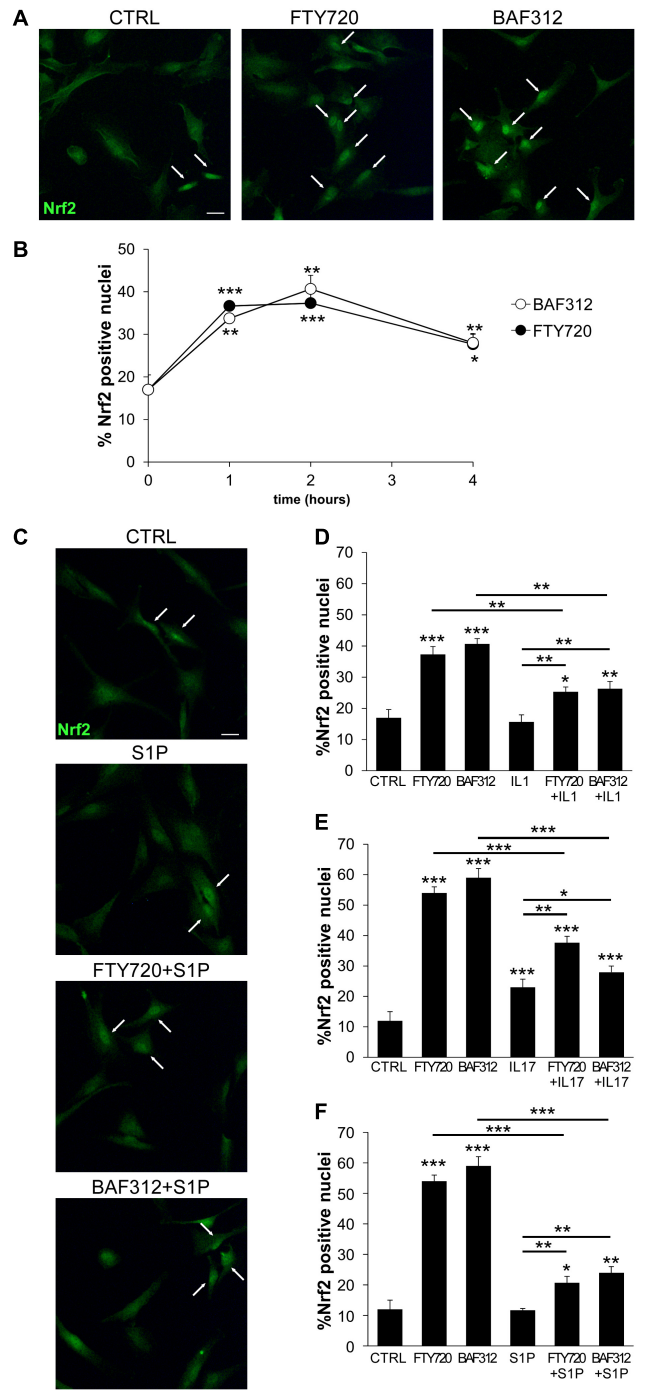

FIGURE 4 | S1P receptor modulators induces Nrf2 activation in glial cells. **(A)** Representative immunofluorescence stainings for Nrf2 in human iAstrocytes exposed for 1 hour to FTY720 or BAF312. **(B)** Frequency of Nrf2 positive nuclei in human iAstrocytes stimulated with drugs at different time points. **(C)** Representative Nrf2 immunofluorescence stainings in iAstrocytes exposed for 1 hour to S1P alone or after 1 hour pretreatment with drugs. **(D–F)** Frequency of Nrf2 positive nuclei in human iAstrocytes treated with drugs alone or in the presence of IL1 **(D)**, IL17 **(E)** or S1P **(F)**. Data are shown as mean ± SD of a representative experiment out of 2–3 independent experiments. In **(A)** and **(C)** white arrows highlight Nrf2 positive nuclei. Scale bar: 30 μm. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

associated with significant effects on lesion enlargement and brain atrophy (18), thus on the neuropathological, degenerative components of SP-MS due to oligodendrocyte injury and axonal degeneration aggravated by dysregulated activity of CNS resident cells (30). Astrocytes may contribute to these processes by several mechanisms, including the release of inflammatory cytokines and cytotoxic factors and the formation of a dense glial scar inhibiting tissue repair (31, 32). *In vivo* findings from experimental neuroinflammation support the hypothesis of protective effects of BAF312 in the CNS (33). In fact, direct administration of the drug into the CNS of animals with experimental MS ameliorates disease expression, and reduces CNS inflammation and loss of GABAergic signaling (33). This action is accompanied by shrinkage of astrogliosis *in vivo*, suggesting an impact on the inflammatory activation of the astrocyte (33). Astrocytes upregulate S1P receptors S1P1 and S1P3 *in vitro* and *in vivo* under inflammatory conditions (7, 34), thus they may become target of S1P signaling modulators. BAF312 may directly induce *in vitro* glial Ca^{2+} levels and ERK phosphorylation mainly via S1P1 receptor (25). Our *in vitro* data corroborate the evidence for BAF312 action on several distinct astrocyte functions, and identify Nrf2 and NFκB as crucial transcription factors regulated by BAF312 in astrocytes. Whilst glial Nrf2 induces *in vivo* anti-oxidant, anti-inflammatory and neuroprotective responses (35), astrocytic NFκB is primarily involved in pro-inflammatory reactions, scar formation and neurodegeneration (36–39). Thus, the balance between these two pathways is critical for regulation of cellular responses to stress and inflammation. It is relevant to underline that functional S1P signaling is necessary for cytokine-evoked astrocyte activation, as its targeting by FTY720 completely impairs glial NFκB translocation in response to IL1 and IL17 (7). Here we show for the first time that the S1P modulator BAF312 rapidly induces Nrf2 nuclear translocation in glia cells, and that this phenomenon is paralleled by the blockade of NFκB activation under inflammatory conditions. Considering that S1P concentration is high in blood (>100 nM) (40) and that levels around 100 nM may be plausibly reached in the inflamed CNS due to blood-brain barrier breakdown, to interfere with S1P signaling we employed 100 nM BAF312 or active FTY720 in our *in vitro* tests. No information is available about the concentration reached by the two drugs in the human CNS, while it is known that BAF312 and active FTY720 concentrations are, respectively, around 60 and 5 nM in plasma of human treated subjects (41, 42). Notably, evidences from mouse models indicate that drug levels in the CNS exceed those in blood severalfold, and that FTY720 accumulates even more in the inflamed vs. healthy CNS (43, 44). Here, we report that BAF312 and FTY720 display comparable efficacy in parallel *in vitro* tests, confirming the crucial pathogenic role of S1P signaling in astrocyte function and implying that the drugs may result equally potent on glial cells, assuming that the levels of active FTY720 are similar to those reached by BAF312.

Although S1P signaling in astrocytes can be triggered by two (S1P1 and S1P3) receptors, S1P1 appears to play a major role in glial functions. In fact, S1P1-selective agonism reduces astrogliosis in experimental MS with similar efficacy to FTY720, which targets several S1P receptors (45). Further, transgenic mice with selective removal of S1P1 from GFAP-expressing cells

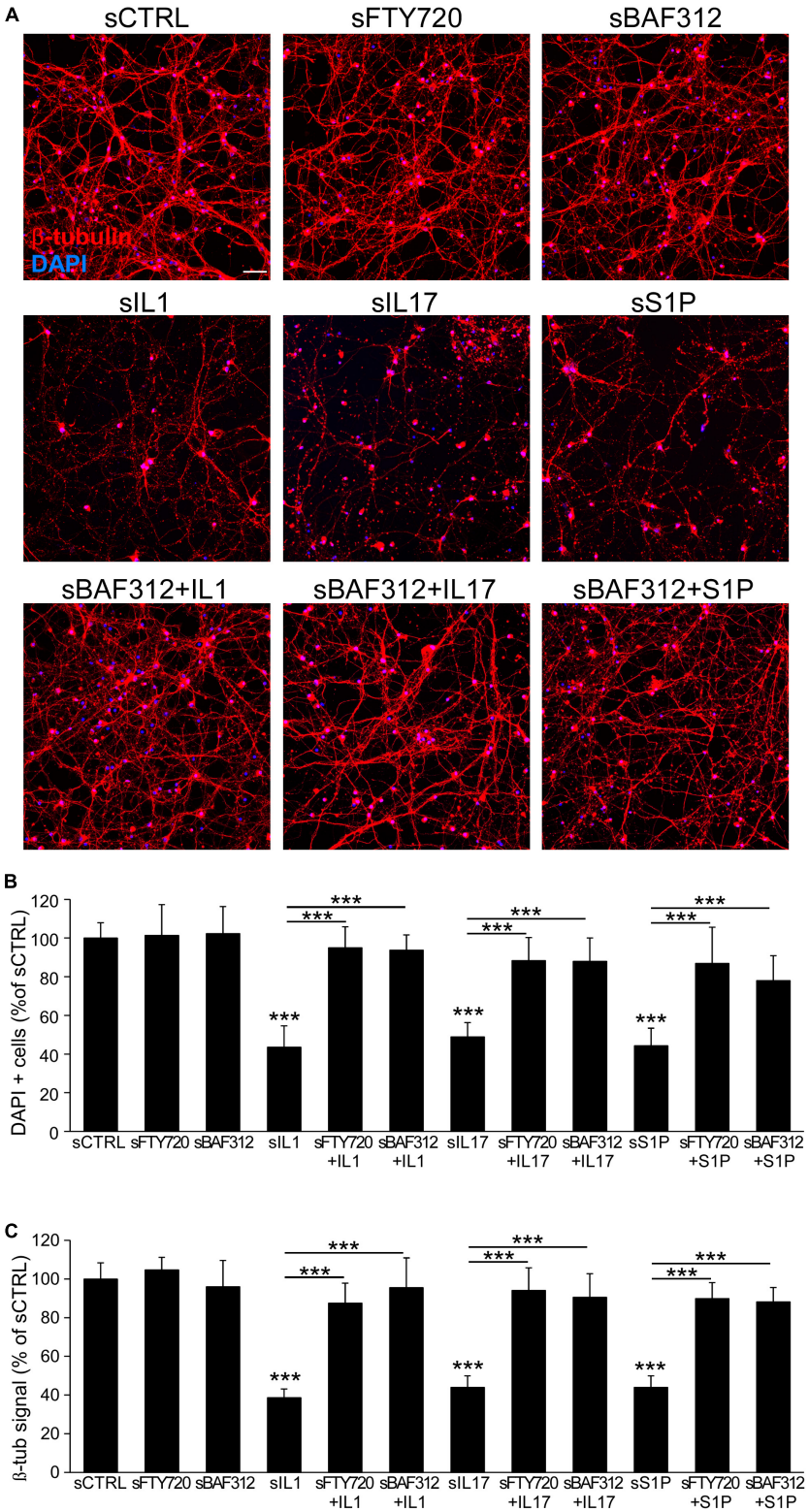

FIGURE 5 | BAF312 blocks neurodegeneration induced by astrocyte responses to cytokines and S1P. **(A)** Representative immunofluorescence stainings for β-tubulin and DAPI of spinal neurons after exposure to media generated from vehicle (sCTRL)-, FTY720- or BAF312- treated astrocytes (upper panels), IL1-, IL17- or S1P-activated astrocytes (middle panels) or eventually from astrocytes treated with BAF312 (lower panels). **(B,C)** Quantification of cell number **(B)** and β -tubulin signal **(C)** expressed as percentage respect to supernatants from control-treated cultures (sCTRL). Graphs show cumulative results from three independent experiments. Data are represented as mean ± SEM. Scale bars: 50 μm. ***$p < 0.001$.

develop milder neuroinflammatory disease and do not respond to FTY720 treatment (45). Here we show that, in our *in vitro* human system, S1P signaling via S1P1 is necessary and sufficient to modulate astrocyte behavior. In fact, glial responses to S1P are efficiently and equally inhibited by BAF312 (which targets S1P1) and FTY720 (which targets both S1P1 and S1P3). Moreover, treatment with the potent and selective S1P1 antagonist NIBR-0213 is sufficient to abolish NFκB activation.

Internalization of S1P1 receptor following ligand binding has been observed in cells artificially overexpressing S1P1 (45, 46). This phenomenon has been visualized in primary rodent astrocytes exposed to nanomolar doses of FTY720 or to micromolar BAF312 (24, 25), while no evidence was available for human astrocytes. Here we show that FTY720 and BAF312 induce formation of intracellular S1P1 aggregates with perinuclear distribution in our cells, thus demonstrating internalization of physiological levels of S1P1 in human glia. Notably, internalized S1P1 receptors may maintain signaling activity following exposure to FTY720 and not S1P, suggesting persistent agonism mediated by the drug (24, 46). On the other hand, our studies indicate that S1P receptor modulators are functional antagonists of S1P and cytokine signaling. This action may thus result either from the loss of surface S1P1 receptor or from the interference of internalized S1P1 activity with inflammatory signaling, an issue which deserves further investigation.

A pathological consequence of neuroinflammation is the dysfunction of glutamatergic transmission due to malfunctioning of glutamate transport. Glutamate is the main excitatory neurotransmitter in the CNS, but excessive glutamate accumulation in the synaptic and extra-synaptic spaces leads to neuronal death through a process called excitotoxicity (47). Under physiological conditions glutamate clearance from the extracellular milieu is primarily achieved by astrocytes via the glutamate transporters GLAST and GLT1 (48), whose levels, however, become low under inflammatory state (49, 50). The beneficial effect of FTY720 in experimental MS has been associated with restoration of glial glutamate transporters (51). Our study proves that inflammatory cytokines and S1P indeed downregulate GLAST and GLT1 proteins in astrocytes, and that BAF312 or FTY720 directly support the maintenance of these transporters. This implies that S1P signaling modulators may restore proper glutamate buffering by astrocytes back to physiological levels.

Astrocyte activation is crucial in driving inflammation-induced neurodegeneration. In fact, S1P- or cytokine-activated astrocytes release factors that trigger neuronal death, as nitric oxide (7) or reactive oxygen species (52). Importantly, blockade of S1P signaling in glia cells by FTY720 hampers NO release in response to S1P and inflammatory cytokines, and prevents from astrocyte-induced neuronal death (7). Similarly to what shown for FTY720 (7), our experiments on primary cultures of spinal neurons demonstrate that neurotoxicity mediated by conditioned media from activated astrocytes is abolished when astrocytes are exposed to BAF312. This final evidence unequivocally confirms that the net result of the modulation of S1P signaling in the astrocyte is indeed the blockade of astrocyte-mediated neurodegeneration.

CONCLUSION

In conclusion, our investigation highlights indirect neuroprotective properties for BAF312 via targeting S1P-S1P1 axis in glia cells.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The participants to the study provided their written informed consent approved by the Ethics Committee of Ospedale San Raffaele, Milan.

AUTHOR CONTRIBUTIONS

EC and CB performed *in vitro* experiments and statistical analyses. AD performed the iAstrocyte differentiation from human iNPCs. FR and LO generated and provided the human iNPCs. EC and CF analyzed the data and wrote the manuscript. GC and GM discussed the project and results. GM provided reagents. CF conceived and designed the experiments. All authors read and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.00635/full#supplementary-material

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REVIEW
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Crosstalk Between Astrocytes and Microglia: An Overview

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Based on discoveries enabled by new technologies and analysis using novel computational tools, neuroscience can be re-conceived in terms of information exchange in dense networks of intercellular connections rather than in the context of individual populations, such as glia or neurons. Cross-talk between neurons and microglia or astrocytes has been addressed, however, the manner in which non-neuronal cells communicate and interact remains less well-understood. We review this intriguing crosstalk among CNS cells, focusing on astrocytes and microglia and how it contributes to brain development and neurodegenerative diseases. The goal of studying these intercellular communications is to promote our ability to combat incurable neurological disorders.

Keywords: microglia, astrocytes, neurons, glia, CNS, neurological disorders, neurodegeneration

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INTRODUCTION

Every organ possesses one cell type whose properties incarnate and define its function. For the central nervous system (CNS), that cell is the neuron. Synaptic communication among neurons is organized in neural circuits, which carry out humanity-defining tasks such as written language, as well as brain function, ranging from breathing to motor behavior to perception. Each of the populations of non-neuronal cells of the adult CNS are remarkably adapted to support neuronal function: astrocytes maintain ionic and neurotransmitter homeostasis, refine synaptic connections, and provide neuronal metabolic substrates; microglia monitor synaptic elements and networks, responding to dyshomeostasis by inducing or removing synaptic elements and by modulating neuronal activity; oligodendrocytes elaborate myelin sheaths, which protect and nourish myelinated neuritic segments. Microglia and astrocytes respond to neuronal injury with programs that include proliferation, morphological alterations, mediator production, and engulfment of cells and subcellular elements. These changes represent the CNS tissue response to damage or degeneration.

During development and early-adult life, forces crafted by evolution optimize the CNS structure and function for reproductive fitness and survival. Given that human life-span now extends well-beyond the end of reproductive capacity, it's axiomatic that, while CNS disorders of aging evoke a tissue response, that reaction isn't shaped by evolution to respond specifically to the challenges posed either by aging or by the ongoing pathogenic process. Research into neuroinflammatory glial biology involves characterizing this tissue response and defining its effects on the outcomes of neurological disorders, as well as searching for therapies to ameliorate injurious glial reactions and restore homeostasis.

As noted above, astrocytes and microglia exert their primary functions toward neurons, and much research addresses the dyadic interactions: microglia-neuron and astrocyte-neuron. It is

also timely to consider how microglia and astrocytes signal to each other, to obtain a more-comprehensive account how their behavior is regulated in the complex context of CNS injury or disease. This review takes the approach of introducing briefly each cell type in relation to its interactions with neurons, followed by a series of "embryonic" illustrating how microglia and astrocytes can communicate. Finally, these interactions will be placed in the setting of varied CNS disorders. In each circumstance, the relevant outcome of astrocyte-microglial communication will be health of the individual neuron or integrity of the neural circuit.

MICROGLIA

More than 100 years ago, in 1919 Pio del Rio-Hortega published an article in which he introduced the term "microglia." He used an improved silver-staining method to delineate microglia and to discriminate them from oligodendrocytes, the other cellular component of the Third Element of the CNS (1). Today we can enjoy the observation of microglia in 3D at nanometer-resolution visualized by volumetric ultrastructural reconstruction using serial block face scanning electron microscopy (SBEM) (2).

Microglial Are Myeloid Cells of the Brain Distinct From Peripheral Macrophages

Microglia are myeloid cells of the CNS parenchyma (3). With regard to ontogeny, microglia differ significantly from their macrophage relatives in other tissues. Both populations come from primitive macrophages, but at the germline level their paths of development diverge. Microglia come from yolk sac erythro-myeloid progenitors and settle the brain early in brain development before the blood-brain-barrier (BBB) closure (4). Migration of microglia progenitors to developing CNS is followed by rapid multiplication and creation of a pool of residual cells that are long-lived and have the ability to renew independently of the hematopoietic system. By the end of second postnatal week microglia become fully matured and express adult gene signature (5). Microglia share some genes with other mononuclear phagocytes, however, several transcripts are highly enriched in microglia, including CX3CR1, P2RY12,13, SOCS3, TREM2, TMEM119, GPR34, and SIGLEC (6). TMEM119, P2RY12, and SALL1 are considered microglia specific markers (5). When settled in the brain, peripheral macrophages possess intrinsic ability to express microglia genes, however the true identity of microglia is the function of both the ontogeny and environment (7). Environmental cues not only reassure microglial identity but also modulate enhancer landscapes in microglia. A recent study by Bennet et al. using a cell transplantation system in mice demonstrated environmental influence on microglial identity and the stunning plasticity of microglial cells (7), which has been further confirmed by Zhan et al. showing that murine microglia have the internal memory of their homeostatic signature, which allow returning to resting state (8). Despite significant changes in gene expression, which have been accompanied by morphological changes induced by *ex vivo* manipulations, after transplantation to the CNS microglia have quickly returned to their normal, homeostatic characteristics. Interestingly, hematopoietic stem cell—microglia like cells (HSC-MLC) that can, after transfer to CNS, imitate microglia, have been found to be enriched in genes associated with neurological diseases such as Alzheimer Disease (AD) (7). Microglia display a broad spectrum of phenotypes depending on environmental assemblage. Attempts to classify microglia as M1 or M2 like in case of other macrophages occurred far too simplistic and have failed, as evidenced by modern transcriptome profiling (9).

Microglia Heterogeneity Is the Most Diverse During the Early Development

Microglia development process is highly dynamic and is characterized by changes in microglial states with unique sets of genes, morphology, distribution, and most likely function (10). Recent studies by Hammond et al. using single-cell RNA sequencing identified several microglial states present in mice brains throughout development, different ages, and conditions including injury (11). In this study, it has been found that the highest diversity of subpopulations of microglia with unique molecularity persist during early development. It has been proposed that unique gene patterns present during development represent specific transcriptional programs rather than the modulation of, already existing, one generic program. Interestingly, one population of microglia has displayed highly activated state despite the absence of any pathology and has been restricted to short postnatal period. Another identified microglial subpopulation has been present throughout life-span with increased prevalence in old age and injury with selective expression of the CCL4 chemokine. This population has also been enriched by expression of other inflammatory signals and has been proposed to be a specialized group to produce inflammatory responses. In general, in early development, microglial have been enriched in genes associated with metabolism, growth, motility, and proliferation and some of them became re-activated during injury and in aging brain. As the brain matures, microglia become less heterogeneous until aging and/or injury, which are characterized, as in development, by a large diversity and immature state.

A recent study by Li et al. has reported exceptional findings of spatiotemporal transcriptomic heterogeneity of microglia and other brain myeloid cells in six different brain areas and through three developmental stages in mice (12). Using deep single-cell RNA-seq technique, which enabled the detection of about three times as many genes per cell with higher detection rates as compared to previous scRNA reports, the study has demonstrated that regardless of the region of the brain, the adult microglia displayed vastly similar transcriptomes. This data has changed our perspective on microglial regional heterogeneity in adult brains based on previously reported findings. However, unlike adult cells, microglia in postnatal brain have been characterized by developmental complexity, with one subpopulation detected in the white matter unique in terms of morphological features such as round and ameboid shape. Interestingly, this newly identified white matter-associated

microglia (WAM) have been found to be transiently present in the first postnatal week and be involved in phagocytosis of newborn oligodendrocytes and, most likely, astrocytes. It has been found that WAMs' activation is not dependent on TREM-signaling.

In the Developing Brain Microglia Are Involved in Neurogenesis and Synaptic Pruning

During brain development, microglia development, and maturation is synchronized with neurogenesis. Neural and microglia cells exhibit extensive, dynamic physical interactions. The development and maintenance of microglia critically depends on the expression of Colony Stimulating Factor-1 Receptor (CSF-1R), which responds to its ligands CSF-1 and IL-34, released in the developing brain by neurons (13). Blockage of CSF-1R leads to microglia elimination and abnormal circuit connectivity in adult mice (14). Further microglia differentiation and maturation depend on TGF-β signaling. In a mouse model of limited CNS TGF-β expression, microglial maturation is altered (15). Microglia guide neuronal development mostly by processes like phagocytosis and factors production such as nerve growth factor (NGF) and tumor necrosis factor (TNF). The brain environment changes constantly during development, which elicits continual changes in microglial states. During late-embryonic and early-postnatal brain development, microglia are implicated in synaptic pruning, which eliminates excess and weaker synaptic connections. In the context of refinement of ipsilateral and contralateral retinogeniculate projections to the dorsolateral geniculate nucleus (dLGN) during the first postnatal week of life in mice, this process involves Complement Receptor 3 (CR3) (16). In this context, presynaptic elements destined for removal are decorated with "eat me" signals in the form of complement molecules C3 and C1q recognized by microglia, the only CNS cells with CR3 expression. Besides complement dependent pruning, mechanisms based on CX3CL1—CX3CR1 is also involved in synaptic elimination and maturation (17, 18). In mice, interference in refining neural circuits by eliminating complement-cascade or fractalkine-receptor signaling leads to circuit or connectivity abnormalities (16, 17). Some developmental processes based on complement dependent phagocytosis by microglia may occur in a gender-specific manner. This is the case in the process of refining dopaminergic circuity during adolescence in the nucleus accumbens. In male rats this process, based on the elimination of the dopamine receptors D1rs, is mediated via microglia C3-phagocytic activity (19). The mechanisms of dopamine receptor elimination in females remain to be determined.

In the Adult Brain, Microglia Participate in Neuromodulation, Synaptic Plasticity, Learning and Memory Formation

In the adult brain, microglia perform many functions as diverse as neuronal support, synaptic modulation, reorganization of neuronal circuitry, and the production of significant amounts of antimicrobial peptides. Microglia communicate with neurons and neighboring cells via neurotransmitter receptors, purinoreceptors and ion channels. ATP is a key communicator of microglia with neurons and a key stimulator for microglial movement toward ATP sources. Using the larval zebrafish model Li et al. demonstrated a reciprocal cell-to-cell communication between microglia and neurons in a neuronal activity dependent manner based on purinergic receptor signaling (20). Activated neurons send "find me" signals (eg., ATP) through pannexin hemichannels to resting microglia. This is a signal for resting microglia to move processes toward targets, surround highly active neurons and consequently suppress neuronal activity.

It has been proposed that ATP signaling to purinergic receptors signals for release of Brain Derived Neurotrophic Factor (BDNF), which plays varied roles in neuronal differentiation, synaptic development, and plasticity. BDNF binds to neuronal TrkB, and regulates synaptic transmission and plasticity in mice, including formation of new synapses during learning (21). In one provocative experiment, deletion of *BDNF* from microglia did not change overall brain BDNF levels, but produced a phenotype showing deficits in a standard motor learning task accompanied by lack of new synapse formation in motor regions (21). In murine and human brain, microglial processes contact neuronal somata at specialized junctions regulated by purinergic signaling in microglia (22). These junctions are perturbed by neuronal injury and are coupled to neuronal mitochondrial activity (22).

An intriguing role is attributed to the neuron-microglia communication based on fractalkine/CX3CL1 produced by neurons and CX3CR1 expression limited to microglia (14). In neurogenesis and spatial learning, this interaction is particularly important because the lack of CX3CR1 reduces neurogenesis and lessens the efficiency of task learning (13). CX3CL1/CX3CR1 signaling has been characterized almost exclusively in mice, although there is an orthologous human chemokine-receptor pair.

Microglia Rapidly Respond to Milieu Changes via Ion Channels, Cell Surface Receptors, and Epigenetic Reprogramming

The brain environment is highly dynamic, especially during development but also during adulthood and requires rapid responses from microglia. In a healthy adult brain in the optic tectum of larval zebrafish, microglia remain branched and scan the environment sensing neuronal activity and neurotransmitters reassuring neuronal proper functioning (20). Using two-photon imaging, a cluster of 100 genes called "sensome" has been discovered, which microglia use to detect changes in the environment. Two genes belonging to microglia sensome, a triggering receptor expression on myeloid cells 2 (TREM2) and CD33, are known risk factors for late-onset AD (14). Microglia sense changes in the environment using the processes that extend toward targets via differentially regulated non-directional as well as directed motility. Microglia processes monitor release of ATP, which is a main attractant and stimulus of microglia, entry of pathogens and fibrinogen, synaptic function, and

activation of neurons. Microglia rapid and reversible responses to environmental changes are possible in part by activation of ion channels and cell surface receptors (23). Activation of ion channels leads to quick alteration of membrane potential that effects ramification, morphology, motility, surveillance, and other microglia functions. It has been found that microglia resting potential, ceaseless surveillance, and interleukin-1β release in murine models is dependent on the tonic activity of two-pore domain channel THIK-1, the main K+ channel, and that this process is independent on purinergic receptor P2Y12 activation (24). In contrast, the direct motility and extending processes toward tissue damage require activation of microglial P2Y12 receptors by ATP, and this process is independent on THIK-1 activity.

Dying neurons and non-functional synapses need fast clearance by microglia to prevent pathology. Microglia clearance phenotype is region specific and is tightly tuned by epigenetic mechanisms. It has been found that in mouse adult brain, striatal microglial phagocytic activity is epigenetically suppressed by the Polycomb repressive complex 2 (PRC2) as compared to the cerebellum (25). The latter requires higher clearance activity and the cerebellar microglia phenotype mimics that found during development and during certain neurodegenerative diseases. Of note, the cerebellum exhibits a spectrum of primary neurodegenerative processes, and recently has been implicated in the pathophysiology of AD although the neuropathological manifestations in cerebellum are less dramatic than those found in the forebrain (26, 27).

Epigenetic signals control microglial function also during the course of development and if altered might lead to neurodegenerative and psychiatric diseases. In mice, prenatal deletion of two class I histone deacetylases, Hdac1 and Hdac2, prominent regulators of epigenetic reprogramming and macrophage inflammatory responses, compromised microglial development. The deletion of these two genes in microglia in a mouse model of AD resulted in reduced amyloid deposition and improved cognitive function (28). It has been found in a mouse model of Alzheimer's disease that peripheral immune stimulation that induces acute immune training and tolerance in the brain might influence epigenetic reprogramming in microglia (29).

Variants of Some Microglia Genes Are Risk Factors for Neurodegenerative Diseases

Microglia as part of the innate immunity respond to wide array of stimuli, including β-amyloid (Aβ), a toxic protein that accumulates in aging brains most likely as a consequence of slowing down Aβ metabolism and microglia phagocytic activity and is partially responsible for AD pathology. Microglia react to injury through morphological changes, increased proliferation, migration to the target, phagocytosis, activation of the NLRP3 inflammasome, and consequently the release of proinflammatory mediators (30). However, direct translations of cytokine functions that are well-defined in periphery may not operate in CNS context, with example of TNF-α displaying neuroprotective properties or TGF-β1 that is upregulated in aging and after CNS injury (6, 31).

All neurological diseases possess some inflammatory component and microglia are important contributors to brain pathology. Large-scale genome-wide association studies (GWAS) in AD model mice allowed for identification of more than 20 loci in immune-related genes associated with risk factors for neurodegenerative diseases with majority of them expressed by microglia or myeloid cells (31). One of the most intensely studied risk factors for neurodegenerative diseases is mutated TREM2, an innate immune receptor expressed by myeloid cells including microglia. During the early stages of brain development in mice, TREM2 plays a key role in elimination of extra synapses by regulation of microglia activity (32). TREM2 and Tyrobp (DAP12) form a signaling pair that suppresses inflammatory responses in mouse microglia *in vitro,* by reducing cytokine production and increases phagocytic activity that might lead to reduction of Aβ deposition and limitation of neurodegeneration. Several studies in mouse models for neurodegenerative diseases demonstrated opposing roles of TREM2 deficiency on Aβ and tau pathologies (two pathological hallmarks of AD) with amelioration of amyloid and exacerbation of tau pathology (33). For example, TREM2 deficiency in mice plays a stage-dependent role in contributing to amyloid deposition (34). TREM2 sustains metabolic fitness, energy homeostasis, proliferation, and survival in mouse microglia through mTOR signaling. TREM2 deficiency in a mouse model of AD causes metabolic and energetic imbalance followed by increased autophagy that resulted in a dysfunctional microglial state (35). R47H variant of TREM2 is one of the strongest single allele genetic risk factor for AD (36, 37). A mouse model of AD heterozygous for the TREM2 R47H allele showed loss of TREM2 function and enhanced neuritic dystrophy around plaques (38). These findings agree with other studies of mouse models and human subjects with R47H TREM2 mutations, consistently finding that microglia surround amyloid plaques, create a putative neuroprotective barrier, and limit plaque-associated neuritic dystrophy (39). This new role for "microglia barrier" in AD pathology has been reviewed (40).

During an injury or disease, microglia display a variety of phenotypes that can be detrimental or beneficial depending on the context (5). Human gene expression profiling obtained from frozen-post mortem AD specimens of superior frontal gyrus using RNA-Seq, has not been found to resemble any disease activation-related gene profile from animal models (41). Instead, this new profile of human Alzheimer's microglia/myeloid cells (HAM) resembled an "enhanced human aging" transcriptomic phenotype. The sole commonality between data obtained from animal models and HAMs involved genes associated with lipid metabolism and lysosomal biology. More data from human subjects are awaited since presently available animal models poorly reflect human pathophysiology. One of many problems concerns the usefulness of young mice with aggressive amyloid deposition phenotypes for studying age-related neurodegenerative diseases such as AD (42).

Cellular therapies with microglia serving as vehicles carrying genes or gene products to the CNS might be promising to confine neurological diseases. Recently, new approaches including usage of induced pluripotent stem cell (iPSC) microglia are potentially

hopeful as therapeutic strategies (43). Microglia-like cells can be efficiently generated and enriched from multiple human embryonic stem and iPSC cells (44) although the *in vitro* context does not support expression of a transcriptome mirroring that seen in acutely isolated cells (45). Soluble cerebrospinal fluid TREM2 shows considerable promise as a biomarker for ongoing CNS pathology in AD (46). Interestingly, higher levels of CSF's TREM2 in comparison to phosphorylated tau is associated with attenuated cognitive decline in AD patients.

ASTROCYTES

Astrocytic Diversity Is Most Pronounced in Humans

Astrocytes (from Greek *astron* means *star*) have gained this name due to their characteristic star-like shape with long processes connecting with almost all types of CNS cells. They represent the largest group of glial cells with one astrocyte touching base with nearly 2 million synapses in the human brain. Unlike neurons, well-preserved among species, human astrocytes have undergone amazing changes during evolution (47), which most likely led to the development of unique human characteristics such as logical thinking and cognition. It has been found that human astrocytes extend 10 times more processes and are four times larger than mouse astrocytes. This correlates with the extreme expression of astrocytic PMP2, a fatty acid binding protein important for the normal structure of membrane lipids. Forced expression of PMP2 in the brains of neonatal mice resulted in an increase in diameter and number of astrocytes (47). Drawings by Ramon y Cajal at the beginning of the 20th century have shown a very complex structure of astrocytes, and current technologies confirm the extreme pleomorphism of astrocytes, especially in the human brain (48). The astrocytic phenotype is more defined by their mutual relations with neurons and the vascular system, then the expression of surface markers. Although glial fibrillary acidic protein (GFAP) is not completely astrocyte specific, for decades this marker has been used to identify astrocytes in the CNS (49). An attempt has been made to characterize astrocyte classes in adult murine CNS using dual staining of GFAP and a calcium-binding protein B (S100b) and nine astrocyte groups have been defined with the conclusion that the astrocytic phenotype is a function of the local microenvironment and operating requirements (50). Although they are highly heterogeneous, traditionally, astrocytes are divided into two major groups based on their location and structure. The first group includes protoplasmic astrocytes in the gray matter with "bushy" appearance and direct contact with blood vessels through their special anchorage at the end-foot. The second group contains fibrous astrocytes present in the white matter, contacting Ranvier nodes, and myelinated axonal pathways, where they support myelination (51).

Astrocytic Ontogeny

Astrocytes are generated in the ventricular zone from the same progenitor cells as neurons and oligodendrocytes, called radial glial cells. Radial glial cells derive from neuroepithelial stem cells. In addition to generating the main classes of brain cells, they also serve as scaffold for localization of migrating neurons within developing brain layers (51). Astrogenesis, emergence of maturing astrocytes from radial glia, begins during mid-embryogenesis and continues postnatally (48). Locally astrocytes divide substantially throughout the first month of life.

Astrocytes Contribute to Formation of Neural Circuits

Synaptogenesis takes place across approximately the same stages of development as does astrogenesis, beginning before many astrocytes are present and continuing postnatally in the presence of increasingly-numerous astrocytes (52). Synapses are interconnecting elements between two neurons that allow the transmission of signals in neuronal networks. It takes commitment from both, the glial cells and neurons to create a functional synapse in which immature neurons guided by astrocytes find partners to make connections (53). The recognition of astrocytes in the formation of synapses and neural circuits have come from experiments with neuronal cell culture showing the inability of isolated neurons to survive and form synapses without the addition of astrocytes or factors that they secrete (54). The astrocytic modulation of synaptogenesis is mediated by contact between cells and secreted factors. Cell-cell contact is particularly important for embryonic neurons to form excitatory and inhibitory synapses and is partially facilitated by cell adhesion molecules present on both parties: astrocytes and neurons. For example, astrocytes express neuroligin that binds to neuronal neurexin, which is important not only for synaptic contact, but also for astrocyte morphology and accurate synaptic function in the mouse cortex (55). Additionally, astrocytes influence the growth and development of synapses by secreting stimulatory and inhibitory mediators associated with synaptogenesis. Astrocytes inhibit synaptogenesis by producing two negative regulators: Brain Derived Neurotrophic Factor (BDNF) and Secreted Protein Acidic, Rich in Cysteine (SPARC). SPARC limits the levels of AMPA receptors (postsynaptic glutamate receptors, whose activation leads to strengthening of the synapse), ultimately modulating the activity-dependent elimination of synapses in mice (56). SPARC antagonizes presynaptic Hevin/SPARCL1, which together with Thrombospondins (TSP1,2) plays an important role in the formation of glutamatergic synapses and provides synaptic stabilization and consolidation. Formation of functionally active synapses is maintained by heparan sulfate proteoglycans, glypican 4 and 6 (Gpc4,6) in mouse models (57). Gpc4 secreted by murine astrocytes acts on presynaptic accumulation of neuronal pentraxin 1 (NP1), which further stimulates active synapse formation by clustering of AMPA receptors rich in GluA1 (58). The increase in AMPA receptors and the reduction of gamma-aminobutyric acid (GABA) receptors present in inhibitory synapses is regulated by astrocytic TNF-α. The presynaptic activity and upregulation of synaptic transmission is partially maintained by cholesterol, lipid synthesized by astrocytes, which in combination with ApoE is transported to neurons. Mice with astrocytes with

interrupted lipid synthesis show impaired synaptic development and plasticity (59).

During synaptogenesis but also in adult mouse brain, synapses are removed in activity-dependent fashion to refine neural circuits. This task is allocated to glia, including microglia and astrocytes (60). In mice, astrocytes utilize Megf10 and Mertk to target synapses for removal by direct engulfment. Astrocytes also contribute to refining neural networks through production of soluble factors. As one example, astrocytes release TGF-β, which increases complement C1q expression in neurons and makes them visible for phagocytosis by microglia (53). Synapse engulfment is also regulated by astrocytic IL-33, a member of the IL-1 family, via IL1RL1 receptor on phagocytic murine microglia (61). Interestingly, microglial TREM2, implicated in risk for neurodegenerative diseases, is required for microglia to signal to astrocytes to limit their synapse uptake. Mice lacking TREM2 show reduced synapses resulting from loss of this regulatory mechanism during development. In TREM2-null mice, high-fat diet during adulthood reignites astrocytic synapse removal (62), showing that astrocyte engulfment of synapses is under active restraint in adult mice.

Astrocytes Guard the Proper Functioning of Synaptic Circuits

In the adult brain, astrocytes continue to guard proper functioning of the brain and neurons. Astrocyte processes are an inseparable part of synapses, and are well-positioned to respond and/or control the concentration of neurotransmitters via specific membrane receptors and/or their uptake by membrane transporters like AMPA and N-methyl-D-aspartate (NMDA) glutamate transporters (63). Glutamate, a major neurotransmitter released by neurons is toxic in excess and its proper synaptic concentration is maintained by astrocytes. Astrocytes take in glutamate, convert it to glutamine and in this form shuttle it back to neurons. Glutamine acts as a precursor for glutamate and GABA. Uptake of glutamate by neurons is partly facilitated by fractalkine, a chemokine produced by neurons that promotes neuroprotection, and this action requires astrocyte-microglia communication, because only microglia express the receptor for fractalkine in CNS. Astrocytes have receptors for neuronal mediators, including G-protein-coupled receptors associated with intracellular calcium Ca signaling (53). Astrocyte activity can be visualized by imaging changes in intracellular Ca2+ levels and it is widely accepted that the dynamic communication between astrocytes and neurons studied in murine models is maintained by purinergic receptors and is fortified by the calcium waves and oscillations (49). This type of signaling is used by astrocytes to control many vital function, such as neuronal synchronization, trophic factors concentration and neurotransmitter uptake, modulation of K+ uptake, vascular size sensing and gene expression, and most likely expression of disease-related molecules (63). Reducing astrocyte calcium signaling in mouse striatum confirmed its functional significance (64). In particular, mice demonstrated a marked phenotype of increased repetitive self-grooming associated with increased GABAergic signaling to astrocytes, and mediated by striatal medium spiny neurons (64).

Elevation in astrocyte calcium levels affects production and release of neuromodulators called gliotransmitters, such as ATP, GABA, glutamate, d-serine, lactate, and TNF-α, which affect the plasticity of neurons and their communication with microglia and endothelial cells (49). TNF-α, at physiological levels and produced predominantly by microglia, is needed for astrocytic glutamate release. However, microglial TNF-α at high concentrations causes excitotoxic effects by suppressing astrocyte uptake of glutamate (65).

Astrocytes Display Wide Array of Homeostatic Functions

Astrocytes are interconnecting units with end feet contacting elements of the BBB, which provide nutrients and oxygen. Astrocytes also interact physically with neurons, which rely on this supply. Proper function of blood-and cerebral fluid-brain barriers are supervised by astrocytes. Astrocytes regulate BBB function in part by secretion of factors that modulate barrier properties in context-dependent fashion (66). The structural components of astrocytic endfeet also mediate interactions with the BBB. In particular, astrocyte endfeet are typified by orthogonal array particles, which contain the widely-expressed potassium channel, Kir4.1, and astrocyte-restricted aquaporin-4 water channels. These components support BBB functions of controlling brain potassium ion and water balance. The levels of reactive oxygen species within CNS are also under astrocytic supervision. Astrocytes and neurons build a strong metabolic connection. Astrocytes are major sources of brain cholesterol, crucial for the composition of neural membranes, and a precursor for signaling molecules. In addition, glucose stored exclusively in astrocytes in the form of glycogen allows the use of lactate as a source of energy not only for neurons, but also for other brain cells (53).

Astrocytes Respond to Insult by Upregulation of GFAP and Hypertrophy

The main role of astrocytes in the brain is to protect from damage to the CNS and to repair the nervous tissue after the injury, so it is not surprising that astrocytes are involved in wide array of neurological disorders. The response of astrocytes in neurological disorders such as trauma, neuroinflammation, and neurodegeneration as a physiological defense response is called astrogliosis. Activated astrocytes are characterized by a different molecular pattern, morphology, and function as compared to their normal counterparts. Extensive GFAP expression is a hallmark of reactive astrocytes. Normal astrogliosis after brain injury is associated with inositol 1,4,5-triphosphate (IP3)-dependent signaling pathway and N-cadherin upregulation (67). Reactive astrocytes are essential for scar formation, inhibition of the spread of inflammatory cells, and repair of blood-brain barrier insults. Recent findings show that scar formation may stimulate axonal regrowth after severe spinal cord injury in adult mice (68). In addition, during astrogliosis after invasive injury in mice, the formation of new neurons and oligodendrocytes from

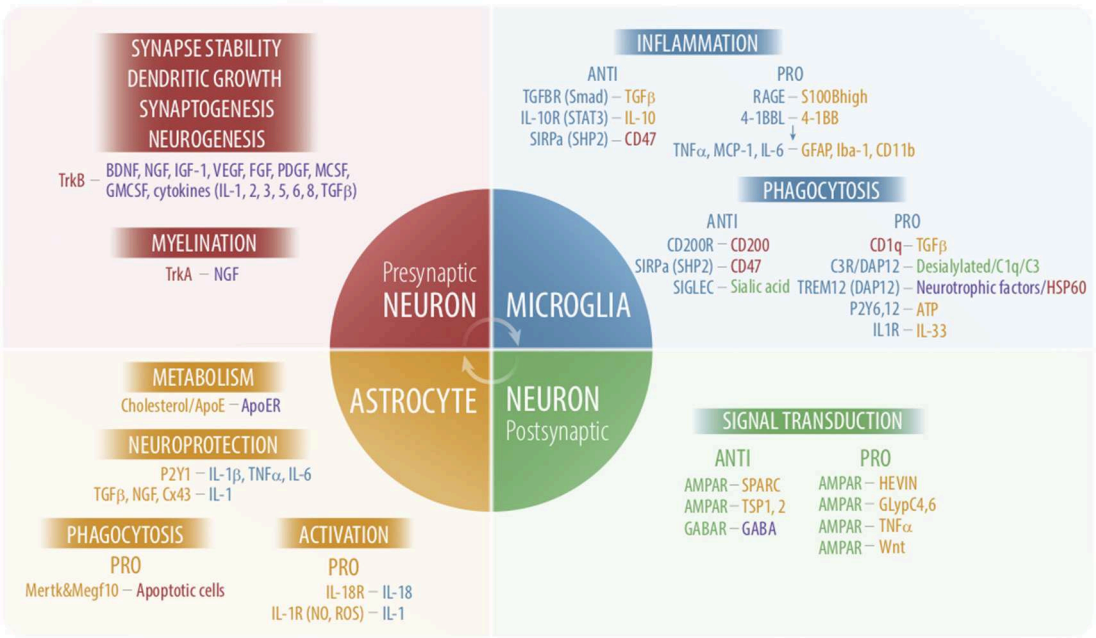

FIGURE 1 | Schematic overview of some interactions among astrocytes, microglia and neurons. Molecules participating in cross-talk and their cellular sources are shown in the same colors. Functions are results of these interactions are depicted in black capital letters next to the cell types where the particular process take place. Purple color reflects multiple sources. AMPAR, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors; APOE, apolipoprotein E; BDNF, brain derived neurotrophic factor; DAP12, DNAX activation protein of 12 kDa; FGF, fibroblast growth factor; GABA, γ-aminobutyric acid; GFAP, glial fibrillary acidic protein; GMCSF, granulocyte macrophage colony stimulating factor; Iba-1, ionized calcium binding adaptor protein 1; IGF-1, insulin- like growth factor-1; MCSF, macrophage colony-stimulating factor; NGF, nerve growth factor; PDGF, platelet-derived growth factor; RAGE, advanced glycation end products; SIGLEC, sialic acid-binding immunoglobulin-type lectins; SIRPa, signal regulatory protein; SHP2, SH2-domain-containing protein tyrosine phosphatase 2; SPARC, secreted protein, acidic and rich in Cysteine; TREM, triggering receptors expressed on myeloid Cells; Trk, neurotrophin receptor tyrosine kinase; TSP1,2, Thrombospondin1,2; VEGF, vascular endothelial growth factor.

stem-like reactive astrocytes has been observed (69). In a healthy brain, astrocytes are organized in non-overlapping domains that can play a role in neuropathology. Reactive astrocytes lost their domain organization in experimental models of epilepsy, but are preserved in the animal model of AD. So far the significance of astrocytic domains in health and disease remains unclear. The effect of reactive astrogliosis in disease is complex: reactive astrocytes can be both beneficial and harmful to surrounding cells and can solve or worsen initial CNS damage. This process has a favorable outcome during acute stress or focal cerebral ischemia, but can limit regeneration at a later stage. Reactive astrocytes may be neurotoxic when producing reactive oxygen species or certain inflammatory cytokines. Local elimination of activated astrocytes improved axonal regeneration after injury in postnatal mice (70). Many chronic neurological disorders are accompanied by chronically stressed, degenerated, and atrophic astrocytes with loss of function, which adds to the progression of the disease. Reactive astrogliosis is a complicated phenomenon, however, it is common in various CNS pathologies. Molecular changes in astrocytes are highly context specific. Although there is a set of genes that are consistently upregulated in various pathologies, about 50% of altered gene expression varies depending on the type of brain damage (67). Unfortunately, at this point, the lack of specific markers for heterogeneous, region-specific astrocyte subtypes significantly limit our understanding of the functional consequence of reactive gliosis in different neurological diseases. In addition, the disadvantage in astrocyte, but also microglial research, is the use of rodent models and *in vitro* settings that poorly reflect conditions prevailing in the human CNS.

ASTROCYTE-MICROGLIA COMMUNICATION

Astrocytes are distributed in a complex network that is connected by gap junctions and are found in all operational areas of the brain and spinal cord and all neuronal layers, and thus bridge and influence neural circuits that are not directly connected. In addition, astrocytes form long processes with the end feet structures that allow communication with blood vessels, another dense multicellular network. Microglia, as revealed by live imaging, are restless cells and constantly move their processes through the brain environment (71, 72). Astrocytes-microglia together with glutamatergic neurons constitute a unit called the "quad-partite synapse," which is necessary for the operation of the circuit and is based on neuro-immune communication (73). Some interactions between astrocytes and microglia in the neuronal context are depicted in **Figure 1**.

Different Ways of Communication Typify Astrocyte-Microglial Cross-Talk

Cross-talk between astrocytes and microglia is maintained in part via secreted mediators, such as growth factors, neurotransmitters and gliotransmitters, cytokines, chemokines, innate-immunity mediators and tissue damage molecules (e.g., ATP), mitogenic factors, NO, ROS, and metabolic mediators such as glutamate, that can be used for cell metabolism and may also mediate tissue changes. In addition, astrocytes, microglia, and neurons communicate via releasing and responding to extracellular vesicles. Extracellular vesicles function over long distances and can contain active biomolecules, including mRNA and miRNA, that are capable of modulating gene expression in distant cells. A proteomic study showed that the extracellular vesicle derived from *in vitro* ATP stimulated microglia were able to induce a molecular reaction in targeted astrocytes (74). Of note, extracellular vesicles may participate in pathogenesis of neurodegenerative disorders by transporting and transferring toxic aggregates, such as tau and Aβ (75). Reduced levels of presynaptic proteins in exosomes derived from neurons have been reported early in disease, and their quantification in patient plasma may carry prognostic and therapeutic value in neurodegenerative diseases (76).

Another route for astrocyte-microglia was described in mice: cross talk may proceed through the gut–brain axis by which metabolites of dietary tryptophan controlled by commensal flora act directly on CNS-resident microglia and their production of Vascular Endothelial Growth Factor-β (VEGF-β) and TGFα, which regulate astrocyte pathogenic activities during inflammation and neurodegeneration (77).

Purinergic signaling though P2Y receptors, expressed in astrocytes and microglia may play a major role in the communication of microglia with astrocytes during the inflammatory response. For example, ATP derived from astrocytes, which binds P2Y12 and P2Y6 expressed by microglia, promotes microglial phagocytosis, and processes extension in rats (78). Binding of ATP by microglia and astrocytes, contingent on which purinergic receptor is expressed, may evoke calcium currents in both cell types, and the production of inflammatory cytokines by cultured dorsal horn microglia (79).

Cytokines Are Important Mediators Between Astrocytes and Microglia

Reactive glia including astrocytes and microglia can express and secrete canonical cytokines such as IL-1β, IL-6, TNF-α, IL-18, TGF-β, and IL-10 after acute tissue injury (80). Contingent on receptor expression, these cytokines function in both autocrine and paracrine manner. They are differentially produced by microglia, astrocytes, oligodendrocyte progenitor/NG2+ cells, and neurons in context-dependent fashion, being expressed when cells sense dyshomeostasis. In the neurotypical context, these cytokines occasionally also play a key roles in physiological processes (81). For example, the IL-33 cytokine of the IL-1 family expressed in developing astrocytes in the spinal cord and thalamus plays a role in synaptic refinement, signaling to microglial IL-1RL1. In gene-targeted mice, IL-33 deficiency results in a surplus of excitatory synapses and a hyper-excitable intrathalamic circuit (61).

In the context of brain injury in mice, cytokines, such as IL-1β, TNF-α, and IL-6 released by microglia, regulate astrocytic responses, and lower astrocyte P2Y1 receptor to enable tissue remodeling and repair (82). By constrast, another set of cytokines produced by activated mouse microglia *in vitro* and composed of IL-1α, TNF-α and complement factor Cq1, induces in mouse astrocytes a putative neurotoxic state astrocytes "A1" (83). Investigation of astrocytes in Huntington Disease (HD) cingulate gyrus using snRNA-Seq, with extensive confirmatory steps for RNA and protein expression, and comprehensive informatics, disclosed three astrocytic states that mapped to transcriptomic clusters (84). This study disclosed no evidence in favor of A1 (neurotoxic) or A2 (neuroprotective) astrocytic states in human neurodegenerative disease.

TGF-β and IL-10 are antagonists to some TNF-α and IL-1 activities and thus participate in regulating the inflammatory response. TGF-β produced by astrocytes signals to microglia among other cells, decreasing expression of some inflammatory mediators. Microglial TGF-β reduces subacute neuroinflammation after stroke in mice (85). Cytokine-activated astrocytes can promote neurogenesis in adult mice in the sub-ventricular zone (86).

Cross-Talk in Disease

As a result of their diverse and complex roles, microglia and astrocytes contribute critically to brain homeostasis, and are now accepted as important disease modifiers.

In the context of inflammatory neurological diseases, cross-talk between astrocytes and microglia seems particularly important. Both microglia and astrocytes are considered to be part of the innate immune system based on their ability to produce immunomodulators and expression of receptors associated with innate immunity, such as complement receptors or Toll-like Receptors (TLRs). For example, the response to LPS requires TLR-4, which is present on innate immune cells and microglia. Although murine *Tlr4* is expressed only in microglia, microglia and astrocytes acutely isolated from human brain both express *TLR4*.

Appropriate astrocyte-microglia cross-talk in disease is necessary for astrocytes to support neuronal survival and function after acute injury. Modeling in mice suggests that microglia constitute a first line of defense, demonstrating activation, and fast recruitment to sites of damage to phagocytose dead cells and debris (87). Secondary to microglial reaction is the activation of astrocytes, which release inflammatory mediators that signal to microglia and can recruit MIG infiltrating hematogenous cells including monocyte-derived macrophages. Reactive astrocytes upregulate GFAP and undergo morphological changes leading to the formation of glial scars, which may limit damage within the affected area (88).

Response in Disease Is Context Dependent

Whether glial cells adopt a phenotype that aggravates tissue injury or promotes brain repair, most likely depends on a basic set of factors, such as the nature of the damaging element, severity and time course of injury, and precise constellation of signals from the environment. The response largely depends on the disease context.

In obesity-induced hypothalamic inflammation of mice, the responses are induced by inflammatory cytokines such as TNF-α, CCL2, and IL-6, and involve canonical gliosis markers (GFAP, Iba-1, and CD11b). This reaction results from direct binding of astrocytic 4-1BB, a member of TNFRSF to its ligand 4-1BBL expressed on microglia. This direct conversation between glia cells promoted monocyte/macrophage proliferation and migration (89). A three-party cross-talk among microglia, astrocytes, and neurons has been identified in the study of viral infection of CNS of mice, via the olfactory route. Protection against the further spread of viral infection has been maintained by an early innate barrier composed mainly of microglia, whose response was regulated by strong IFNAR signaling from neurons and weaker signaling from astrocytes (90).

In summary, glial cells regulate and control each other's function, migration and reactions. A noticeable bi-directional conversation between astrocytes and microglia is evident in the context of neurological disorders. The astrocytes-microglia interplay may determine the phenotype that astrocytes and microglia express during disease. Current therapies for the treatment of neurological disorders and clinical trials based on blocking inflammatory reactions are manifestly insufficient. It is useful to maintain awareness that the CNS environment implicates astrocytes and microglia in programs and functions that cannot be understood in the context of typical immunological reactions. The languages of microglia and astrocytes will be the key to understanding this complex system composed of cells of unimaginable diversity and plasticity, and of manifold yet-unknown functions.

CONCLUSIONS AND FUTURE PROSPECTS

We find ourselves in an unprecedented era of progress in understanding neuroscience and, along with it, glial biology. These insights extend seamlessly from development through aging and disease, which can now be regarded as experiments of nature. The drivers of this progress extend from optogenetics and novel imaging techniques through germline genetics, multi-omics and bioinformatics, through innovative cell and organoid models. The methods are deployed by an increasingly diverse, committed and interactive community of researchers, many of whom arrived from other disciplines as far afield as mathematics, physics, and immunology. Comprehension of the molecular interactions among cells in tissues and organs will lay the biological foundation to identify the right drug targets and modalities for successful assault on disorders of the developing, injured, infected, or aging nervous system.

AUTHOR CONTRIBUTIONS

AM wrote the first draft, performed data mining, generated the figure, and contributed to editing. RR conceived the topic and outline of the review, recruited AM to participate, supervised the preparation of the drafts and figure, and performed final edits. All authors contributed to the article and approved the submitted version.

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The Role of Microglia and the Nlrp3 Inflammasome in Alzheimer's Disease

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Alzheimer's disease (AD) is the most prevalent form of late-onset dementia. AD affects the health of millions of people in the United States and worldwide. Currently, there are no approved therapies that can halt or reverse the clinical progression of AD. Traditionally, AD is characterized first by the appearance of amyloid-β (Aβ) plaques followed by the formation of intraneuronal neurofibrillary tangles (NFTs) composed of hyperphosphorylated tau (p-tau). These lesions are linked to synapse loss and eventual cognitive impairment. Additionally, microgliosis is consistently found in regions of the brain with AD pathology. The role of microglia in AD onset and progression remains unclear. Several recent reports indicate that the assembly of the multi-protein complex known as the NOD, LRR, and pyrin-domain containing 3 (Nlrp3) inflammasome by microglia results in apoptosis spec-like protein containing a CARD (Asc) spec formation, which then nucleates new Aβ plaques, thus amplifying Aβ-associated pathology. NFTs can also activate the Nlrp3 inflammasome leading to enhanced tau-associated pathology. Here, we will review the role of microglia and the activation of the inflammasome in the innate immune response to AD.

Keywords: Alzheimer's disease, microglia, Nlrp3 inflammasome, neuroinflammation, neurodegeneration

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INTRODUCTION

Alzheimer's disease is the most common form of dementia. AD results in neuronal death likely caused by an accumulation of senile plaques primarily composed of amyloid-β (Aβ) peptides, first observed by Alois Alzheimer (1). Plaques promote an environment conducive to forming intraneuronal tau aggregates known as neuritic plaque tau, (NP) tau, and in more advanced stages of AD, neurofibrillary tangles (NFTs) (2, 3). Recent evidence suggests that neuroinflammation, mediated through increased levels of pro-inflammatory products released from innate immune cells, e.g., microglia, contribute to AD, and precedes Aβ plaque deposition and AD onset (4, 5). Microglial dysfunction caused by prolonged amyloid-induced microglial activation may also contribute to AD (6). Microglia are also crucial for maintenance and upholding homeostasis within the brain (7). Upon activation in numerous pathological conditions, including AD, microglial function, and morphology change dramatically (7). Monomeric and oligomeric forms of Aβ as well as tau aggregates such as NFTs activate microglia in AD (**Figure 1**). Additionally, activation of pattern recognition receptors (PRRs) expressed by microglia can influence AD pathology (8). Microglia and other innate immune cells express several toll-like receptors (TLRs), which when activated, subsequently result in the activation of NF-κB and the production of

pro-inflammatory cytokines (9). Additionally, microglia also express several intracellular PRRs that are not membrane-bound such as nucleotide-binding domain and leucine-rich repeat-containing receptors (NLRs) family of receptors, absent in melanoma 2 (AIM2)-like receptors (ALRs) family of receptors and the tripartite motif-containing (TRIM) family member pyrin are known to initiate the assembly of the multi-protein complex inflammasome (10, 11). In most cases the inflammasome complex contains apoptosis spec like protein containing a caspase recruitment domain (Asc), and is also known as an Asc spec (12).

In this review, we will primarily discuss the role of inflammasome activation by microglia in Alzheimer's disease with a special focus on Nlrp3 inflammasome activation.

INNATE IMMUNE RESPONSE IN AD

Microglia originate from primitive macrophages that exit the yolk sac and colonize the neuroepithelium and are the primary immunocompetent cells found within the brain (13, 14). In a normal physiological state, microglia play a critical role in multiple developmental events within the central nervous system (CNS) such as the establishment of neural circuits, synaptic pruning and remodeling, and neurogenesis (15–21). Microglia are also responsible for clearing cellular debris and aggregate-prone proteins including Aβ as well as harmful bacteria and viruses through phagocytosis in diseased states (22). Microglia within a normal adult mouse brain are highly dense in gray matter areas including the hippocampus, basal ganglia, substantia nigra, and olfactory cortex (23). Throughout these areas, the number and role of microglia is highly regulated by the local microenvironment and their interactions with surrounding cells such as neurons, astrocytes, and oligodendrocytes (24).

Studying key Alzheimer's risk genes has provided critical insights into the function of microglia and how microglia modulate pathology in AD. It is known that several genes, expressed exclusively by microglia in the brain, such as CD33, a sialic-acid-binding immunoglobulin-like lectin (SIGLECs), and triggering receptor expressed on myeloid cells 2 (Trem2) carry single nucleotide polymorphisms (SNPs) that influence the risk for developing AD (7). CD33, a PRR on the cell's surface that recognizes sialylated glycoproteins and gangliosides, promotes Aβ deposition and plaque formation (25). Griciuc et al. (25) also found that CD33 expression impedes microglia's uptake and clearance of amyloid-β 42 (Aβ42), resulting in a larger plaque burden. Inhibition of CD33 using a subtype-selective sialic acid mimetic mitigates Aβ plaque-associated pathology by increasing Aβ plaque phagocytosis (26). Trem2 is another microglial surface receptor, it binds phospholipids and other polyanionic ligands and detects changes in the lipid microenvironment (27). Studies have shown that Trem2-deficient AD mouse models exhibit decreased clustering of microglia around plaques and increased neuritic damage, suggesting that this gene is crucial for containing early plaque diffusion (28, 29). Conversely, Trem2 expression in response to tau has been shown to enhance AD-like pathology (30, 31). So although it appears that Trem2 is important in enhancing microglial responses during AD-associated pathology it remains controversial whether Trem2 expression is overall beneficial or pathogenic in AD (29, 31–34). It is also important to note that many of these findings still require replication in patient samples to confirm the roles of these molecules in AD pathogenesis.

Microglia form a lattice throughout the brain and express an array of PRRs, which sense changes in the brain's environment through the detection of danger-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs) among other stimuli (10). When a shift in the microenvironment is detected, these receptors send converging signals that promote a spectrum of microglial responses from surveillance to activation (35). As microglia survey their environment they rapidly extend and retract their filopodia-like processes within an area of the parenchyma, allowing them to survey that microenvironment (35). When activated, microglia adopt different morphologies, and produce various cytotoxic molecules including pro-inflammatory cytokines and inflammatory mediators consisting of nitric oxide (NO) and reactive oxygen species (ROS) (22, 36, 37). In summary, elucidating the links between innate immune activation and microglia's inflammatory responses concomitant with inflammasome activation, is becoming a crucial research area to better understand AD pathology and to find new therapeutic targets that could impede or slow its progression.

NLRP3 INFLAMMASOME ACTIVATION

Several inflammasomes have been implicated in neurodegenerative diseases, the NOD-, LRR-, and pyrin domain-containing 3 (NLRP3) inflammasome in particular has been shown to play a key role in the development and progression of Aβ-plaque formation as well as tau-induced pathology, which has been demonstrated in both post-mortem AD patient brain tissue and *in vivo*/*in vitro* transgenic mouse studies (37–42). The inflammasome is a multimeric protein complex that is most commonly composed of a sensor, an adaptor, and the downstream effector caspase-1 (12). Each inflammasome is named according to the sensor molecule that initiates activation and is activated through two signals that first prime and then activate the complex (43) (**Figure 1**). Upon activation, Nlrp3, and the majority of other structurally related receptors such as other NLRs, AIM2, or pyrin can form homotypic PYD-PYD or CARD-CARD interactions with the adaptor Asc (apoptosis-associated spec-like protein containing a caspase activating and recruiting domain) (44, 45). The interactions between these molecules are composed of an N-terminal pyrin domain (PYD) and C-terminal caspase-activation and recruitment domain (CARD) and result in the formation of a ring-like perinuclear complex called an Asc "spec," a typical indicator of canonical inflammasome activation (12, 46). Following inflammasome activation, Asc recruits procaspase-1 through interactions with the CARD domain of caspase-1 (47). Procaspase-1 is then converted into

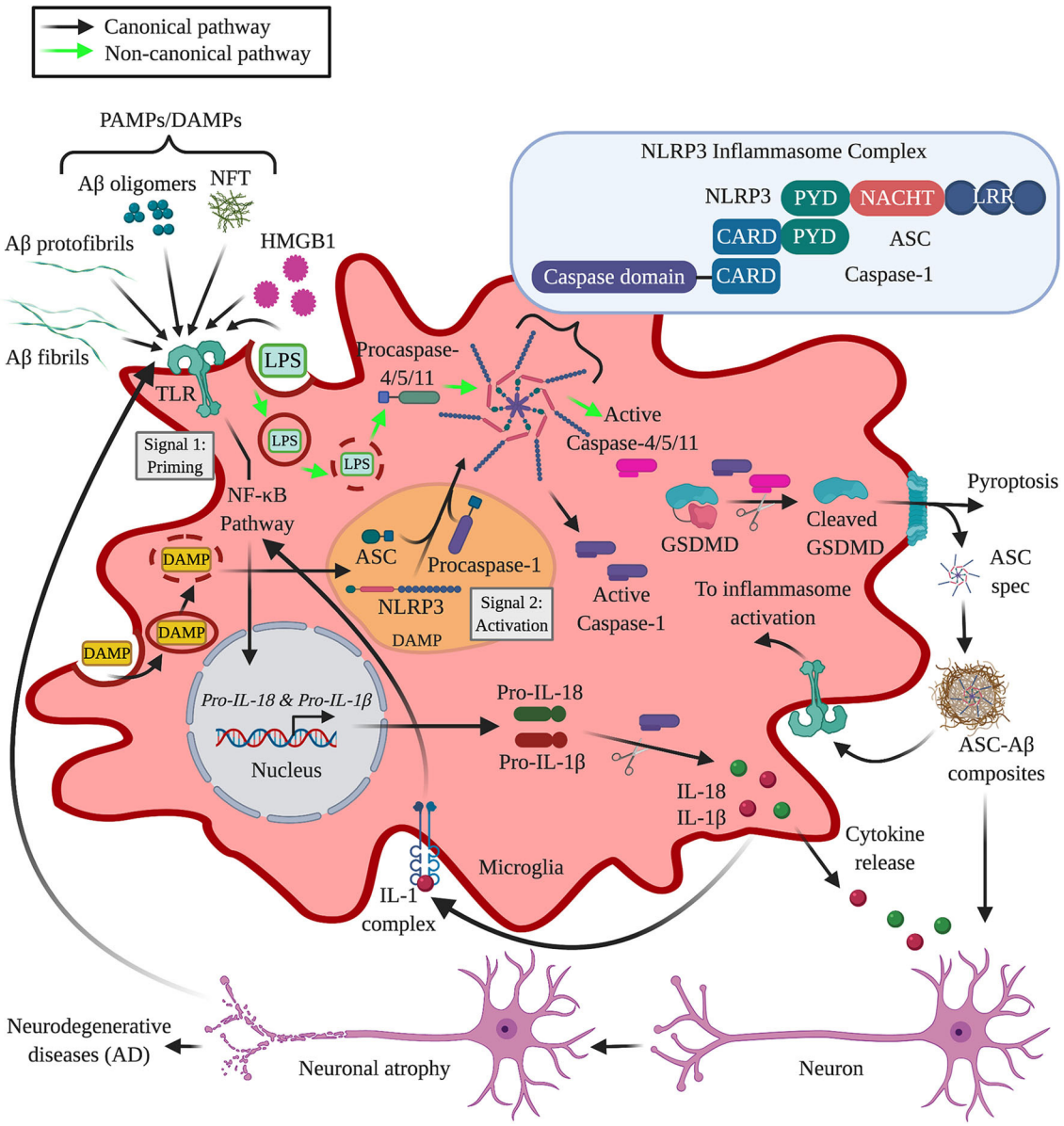

FIGURE 1 | Role of microglia and canonical NLRP3 inflammasome in Alzheimer's disease. Cytokines, pathogen-associated molecular patterns (PAMPs), or danger-associated molecular patterns (DAMPs) bind cell surface receptors [e.g., toll-like receptors (TLRs)] on the microglia, leading to activation of the nuclear factor-κB (NF-κB) pathway (signal 1). The activation of the NF-κB pathway promotes a signaling cascade, resulting in the expression of cytokines such as pro-IL-1β and pro-IL-18 as well as NLR family pyrin domain containing 3 protein (Nlrp3). Through the canonical activation pathway, Nlrp3 oligomerizes in response to an internalized, cytosolic danger signal (DAMP) (signal 2) then recruits Asc and procaspase-1, resulting in inflammasome assembly (Asc-spec assembly) and caspase-1 autoactivation. Activated caspase-1 then cleaves pro-IL-1β or pro-IL-18 and gasdermin (GSDMD). GSDMD cleavage is also induced by caspases 4/5/11 through a non-canonical activation pathway that detects internalized, cytosolic LPS, and other PAMPs/DAMPs that bypass membrane-bound pattern recognition receptors such as TLRs. Subsequently, GSDMD induces pyroptosis, presumably releasing Asc-specs that cross-seed extracellular amyloid beta (Aβ) plaques and creates Asc-Aβ composites that induce a feed-forward cycle amplifying the proinflammatory response. In turn, proinflammatory cytokines including IL-1β and IL-18 induce neuronal damage and death, causing neurodegeneration. Degraded neurons can then trigger a feedback loop by activating microglia. IL-1R complexes are also activated, creating a positive feedback loop that drives additional pro-IL-1β production and primes local microglia for inflammasome activation. AD, Alzheimer's disease; IL, interleukin; NFT, neurofibrillary tangles; ASC, apoptosis-associated spec-like protein containing a CARD; CARD, caspase-activation and recruitment domain; LRR, leucine-rich repeat; NACHT, nucleotide-binding oligomerization domain; PYD, Pyrin domain; HMGB1, high mobility group box 1; LPS, lipopolysaccharide; NF-κB, nuclear factor kappa light chain enhancer of activated B cells created with BioRender.

its bioactive form through proximity-induced autocatalysis, producing mature caspase-1 that cleaves pro-IL-1β and pro-IL-18 into their respective secreted forms (10, 48). Caspase-1 also triggers the cleavage of pore-forming Gasdermin D (GSDMD), which induces a lytic, pro-inflammatory form of cell death called pyroptosis (49, 50). Generally, priming and activation of

the inflammasome occurs in response to two different signals, however, it is possible that one molecule can deliver both signals. For example, LPS can initiate both the formation of the canonical and non-canonical Nlrp3 inflammasome involving human caspases 4/5 and mouse caspase-11 rather than caspase-1 (51–53). Non-canonical Nlrp3 inflammasome activation serves as another layer of defense for pathogens that have evolved to bypass membrane-bound PRRs such as TLR4 (54) (**Figure 1**). This form of activation is prompted by caspases' 4/5/11 detection of cytosolic lipopolysaccharide (LPS), which induces pyroptotic cell death through GSDMD cleavage (43). In AD, the Nlrp3 inflammasome is responsible for the maturation of caspase-1, which is in turn responsible for the maturation and secretion of pro-inflammatory cytokines such as IL-1β and IL-18 that can activate signaling pathways resulting in neuroinflammation and neuronal death (5, 12).

NLRP3 INFLAMMASOME IN NORMAL AGING

The concept of "inflamm-aging," the low-grade chronic inflammatory state that accompanies aging, is a recent topic of interest (55). During aging, inflammasome activation can be triggered by local microenvironment changes associated with aging microglia (56). For example, aging microglia exhibit altered cytokine production, making microglial cells more susceptible to adopting a pro-inflammatory state that also primes the cells for inflammasome activation (56, 57). Aging microglial cells also have an increased accumulation of lipofuschin that has been associated with increased oxidative stress, which may cause microglia to lose their neuroprotective potential and contribute to age-related pathology (58, 59). Additional evidence suggests that components of the inflammasome including caspase-1, caspase-11, Asc, and IL-1β are increased in the cytosolic fraction of hippocampal lysates in aged mice, suggesting that inflammasome formation contributes to inflammation in aging (60). Consistent with the inflamm-aging hypothesis, Youm et al. found that reducing the Nlrp3 inflammasome-dependent pro-inflammatory cascade alleviated age-associated degenerative changes across multiple organs (61). This study also showed that *Nlrp3* gene expression was lower in younger microglia compared to their senile counterparts (61). Thus, the status of inflamm-aging in the brain may be associated with changes in aging microglia prompted by local microenvironment and systemic environment states that induce inflammasome activation.

NLRP3 INFLAMMASOME AND THE MICROBIOME

Emerging evidence has highlighted cross-talk interactions between the gut microbiome and the brain (62, 63). In AD, the composition of gut microbiota can influence the development of or exacerbate the pathology associated with AD (64, 65). The role inflammasomes play within the gut-brain crosstalk is less clear. A newly published study that transplanted gut microbiota from AD patients to either APP/PS1 mice, a double transgenic mouse that carries chimeric mouse/human amyloid precursor protein (APP) and human presenilin 1 (PS1) mutations associated with familial AD, or wild type mice, demonstrated that the transplantation of the gut microbiome of an AD patient can influence AD pathology and Nlrp3 inflammasome activation. This study found that APP/PS1 mice receiving a transplant of the gut microbiome from an AD patient had increased expression of Nlrp3 in their intestinal tract and increased levels of inflammatory factors such as IL-1β and IL-6 in their peripheral blood (62). These mice also exhibited more severe cognitive impairment compared to those that did not receive the transplant. When the gut microbiome from an AD patient was transplanted into wild type mice, the intestinal expression of Nlrp3 was also upregulated but their cognitive abilities were not significantly altered (62). The microglia in the hippocampi of these mice, however, were still activated and there was still an up-regulated expression of inflammatory factors. Taken together, these studies indicate that gut microbiota modulate inflammatory responses through Nlrp3 inflammasome signaling.

NLRP3 INFLAMMASOME IN AD

Both the accumulation and deposition of Aβ as well as NFT formation are detected by cytosolic PRRs, prompting Nlrp3 inflammasome activation in microglia (37, 38). The association between microglial Nlrp3 inflammasome activation and fibrillar Aβ was first demonstrated *in vitro* by Halle et al. (66). Their study found that exposing Aβ fibrils to primary mouse microglia induces IL-1β secretion in an Nlrp3-specific manner (66). Recently, soluble Aβ oligomers and protofibrils have also been shown to induce Asc spec formation in primary microglia cells collected from wild type mice (67). Nlrp3 activation has also been linked with tau aggregates in PS19 mice, a mouse model that overexpresses the human tau protein carrying the P301S mutation (39). The association between Nlrp3 inflammasome activation and tau exacerbates and drives tau pathology in AD mouse models (38, 39). Related work by Ising and colleagues was the first to suggest that the Nlrp3 inflammasome forms a link between Aβ plaques and NFTs (38). They showed that Tau22 mice receiving an intracerebral injection of fibrillar Aβ-containing APP/PS1 brain homogenates exhibited increased levels of tau hyperphosphorylation, cleaved caspase-1, IL-1β, and Asc in cerebral samples as well as significantly higher levels of extracellular Asc specs, which have been shown to seed Aβ plaques (37, 38). However, when they injected the same homogenate in Tau22/$Asc^{-/-}$ or Tau22/$Nlrp3^{-/-}$ mice, tau hyperphosphorylation did not occur and there were lower levels of cleaved caspase-1, IL-1β, and reduced Asc spec formation and release, further verifying that Nlrp3 activation is essential in the Aβ-tau cascade (38). These findings demonstrate a link between both tau and Aβ pathology and confirm Nlrp3 inflammasome activation in Tau22 mice. Additionally, Heneka *et al.* used *Nlrp3*-deficient and *caspase-1*-deficient APP/PS1 mice to show that mice unable to activate the inflammasome were spared from memory deficits and LTP suppression unlike the APP/PS1 mice that exhibited severe deficits in spatial memory formation

(68). Their findings showed that Nlrp3 inflammasome activation restricts beneficial microglial clearance functions while *Nlrp3*- or *caspase-1*-deficiency increases microglial plaque phagocytosis (68). Interestingly, new findings support the notion that caspase-1 activation and pro-inflammatory cytokine secretion precede AD pathology, implying that Nlrp3 inflammasome activation is an early pathogenic event in AD (4, 69). Overall, these data indicate that targeting the Nlrp3 inflammasome in human clinical trials are warranted to determine whether inhibition of the inflammasome will hinder Aβ deposition and NFT formation and correlate positively with cognitive outcome measures.

NLRP3 INHIBITION AS A THERAPEUTIC INTERVENTION FOR AD

As Nlrp3 inflammasome activation through both canonical and non-canonical pathways has been shown to play an important role in the pathology of AD, the Nlrp3 inflammasome has emerged as a possible target for future pharmacological therapies (67, 70, 71). Since Nlrp3 inflammasome activation is a multi-step process, inhibiting Nlrp3 inflammasome activation can be accomplished through several different means including: suppressing molecules that promote inflammasome activation or formation, silencing upstream signals, or by directly or indirectly inhibiting the inflammasome complex formation depending on the molecule targeted (10, 72). Some of the direct inhibitors, which specifically target Nlrp3-Nlrp3, Nlrp3-Asc, or NEK7-Nlrp3 interactions, are ginsenoside Rg3, oridonin, and tranilast (73–75). Ginsenoside Rg3, isolated from *Panax ginseng*, specifically blocks IL-1β secretion and caspase-1 activation by inhibiting LPS priming and Nlrp3 inflammasome assembly (73). In contrast, oridonin, derived from *Rabdosia rubescens*, blocks inflammasome assembly and activation by hindering the NEK7-Nlrp3 interaction, which is crucial for Nlrp3 oligomerization and Asc recruitment to Nlrp3 (74, 76). Tranilast, a historical anti-allergic drug used in the clinic, directly suppresses Nlrp3 inflammasome assembly by blocking Nlrp3 oligomerization (75). Examples of indirect inhibitors include β-hydroxybutyrate (BHB), MCC950, glyburide, and 16673-43-0, a glyburide analog that has no effect on insulin (77–80). BHB hinders inflammasome formation by inhibiting K^+ efflux, which causes mitochondrial damage and exposure to a mitochondrial-specific phospholipid, cardiolipin, that leads to Nlrp3 activation (77, 81). MCC950, on the other hand, selectively blocks both the canonical and non-canonical Nlrp3 inflammasome activation pathways by impeding the ATP hydrolysis motif (80). Glyburide works by suppressing ATP-sensitive K^+ channels and caspase-1 activation while its analog, 16673-43-0, achieves inhibition by inducing conformational changes in the inflammasome following Asc's activation or aggregation (79, 82).

Additional methods of inhibiting Nlrp3 inflammasome activation using autophagy-inducing treatments and microRNAs have recently become possible. Some studies have shown that autophagic proteins such as autophagy-related protein 7 (ATG7), microtubule-associated protein 1 light chain 3B (LC3B), and beclin-1 regulates Nlrp3 inflammasome activation by sustaining mitochondrial integrity (83, 84). This data demonstrated that deficiencies in autophagic proteins such as LC3B increases caspase-1 cleavage, Asc spec formation, and IL-1β release in macrophages (83, 84). Thus, by administering autophagy-inducing agents including resveratrol and cannabinoid receptor 2 (CB2R) agonists such as HU-308, Nlrp3 inflammasome activation can be inhibited (85, 86). MicroRNA based post-transcriptional Nlrp3 regulation also prevents inflammasome formation by reducing endogenous Nlrp3 protein levels (87, 88). MicroRNAs are small, conserved single stranded noncoding RNAs that post-transcriptionally regulate gene expression. They bind untranslated regions (UTRs) of transcripts and modify the stability and translation of the target mRNA, producing an inhibitory effect (89). One such example is miR-223, which targets a binding site in the Nlrp3 3′-UTR and was validated *in vitro* in macrophages (87). This study found that miR-223 overexpression inhibits Nlrp3 protein accumulation and IL-1β production from the inflammasome (87). Although each inhibitor has its own mechanism, they all have a similar effect resulting in decreased inflammasome formation, cytokine release, and systemic inflammation.

Within the context of Alzheimer's disease, some of these inhibitors have already been shown to reduce AD-associated pathology (**Table 1**). For instance, JC-124 and oridonin, two direct Nlrp3 inflammasome inhibitors, have been shown to reduce amyloid burden and microglial activation in AD mouse models (71, 91). Oridonin treatment also showed beneficial effects in attenuating disease pathology by decreasing inflammatory cytokine release in the hippocampus (91). Another study that elevated plasma ketone body levels through an oral dose of medium-chain triglycerides to individuals with AD or mild cognitive impairment reported a significant increase of BHB in serum levels and some subjects exhibited cognitive improvement (93).

TABLE 1 | Potential inhibitors of the Nlrp3 inflammasome.

Type	Nlrp3-targeting Agent	Tested in AD	Neuroprotection Observed	Sources
Direct Inhibitors	Ginsenoside Rg3	N	–[a]	(73, 90)
	JC-124	Y	Y	(71)
	Oridonin	Y	Y	(91)
	Tranilast	N	–	(75)
Indirect Inhibitors	Ketone bodies (i.e., BHB)	Y	Y	(92)
	MCC950	N	–	(80)
	Glyburide	N	–	(82)
	16673-43-0	N	–	(79)
Autophagy-inducing	Resveratrol	N	–	(86)
	HU-308	N	–	(85)
MicroRNAs	miR-223	N	–	(87)
	miR-9	N	–	(88)

[a] *Neuroprotection observations were not applicable in these studies.*

While these findings seem promising, there are some limitations to consider when employing these agents as a potential therapeutic. For example, unintended immunosuppressive effects may result from the use of inhibitors that target IL-1β secretion or signaling (94, 95). Likewise, inhibitors focused on reducing cytokine secretion alone does not address Nlrp3-induced pyroptosis, which may contribute to additional pathology (72). Finally, compounds that inhibit cytokine secretion will not necessarily mitigate pathology driven by Asc spec formation. Continued Asc spec formation will result in further seeding of extracellular Aβ plaques, amplifying amyloid pathology, and the pro-inflammatory response (37). Overall, these studies suggest that hindering inflammasome assembly is a potential intervention method for attenuating AD pathology with the caveat that inhibition of the inflammasome can have severe unintended results.

CONCLUDING REMARKS

Traditionally, the cognitive decline associated with AD was attributed to the accumulation of amyloid and tau but emerging evidence suggests that neuroinflammation driven by and triggering additional Nlrp3 inflammasome activation is another critical contributor to AD pathology. Additional investigations are needed to gain insight into the contribution of other inflammasome pathways in neurodegeneration. Additional research is also needed to further clarify the complexity of microglia's signaling cascades and to determine how to modify the microglial response as a potential method for managing or treating AD.

While regulation of Nlrp3 inflammasome assembly and activation may be a potential therapeutic approach, it is currently unknown whether targeting inflammasome activation in AD will result in a beneficial or detrimental effect on clinical outcomes such as cognitive measures. It is also important to consider other therapeutic approaches aimed at modifying microglial function. It is possible that the pro-inflammatory response reproduced in many experimental studies and human genetic or RNA sequencing studies may be the product of a secondary pro-inflammatory response from tissue injury induced by neuronal death, the spreading of AD pathology, or glial dysfunction. Considering that there is microglial dysfunction in AD such as metabolic defects as well as decreased microglial motility and chemotaxis, promoting normal microglial function may prove an alternative therapeutic approach (6, 96, 97). Additional approaches may also include inhibiting microglial proteolytic cleavage of P2RY12, a G-coupled protein receptor located on glial cells, whose inhibition has been associated with neuroprotective effects and promoting other anti-inflammatory cytokines such as IL-4 or progranulin, which still remain to be tested and compared to inflammasome inhibitors (98–100).

AUTHOR CONTRIBUTIONS

KH conceived the review. KH and TU discussed and contributed to the writing of this review. All authors contributed to the article and approved the submitted version.

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Inhibition of IL-34 Unveils Tissue-Selectivity and Is Sufficient to Reduce Microglial Proliferation in a Model of Chronic Neurodegeneration

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The proliferation and activation of microglia, the resident macrophages in the brain, is a hallmark of many neurodegenerative diseases such as Alzheimer's disease (AD) and prion disease. Colony stimulating factor 1 receptor (CSF1R) is critically involved in regulating microglial proliferation, and CSF1R blocking strategies have been recently used to modulate microglia in neurodegenerative diseases. However, CSF1R is broadly expressed by many cell types and the impact of its inhibition on the innate immune system is still unclear. CSF1R can be activated by two independent ligands, CSF-1 and interleukin 34 (IL-34). Recently, it has been reported that microglia development and maintenance depend on IL-34 signaling. In this study, we evaluate the inhibition of IL-34 as a novel strategy to reduce microglial proliferation in the ME7 model of prion disease. Selective inhibition of IL-34 showed no effects on peripheral macrophage populations in healthy mice, avoiding the side effects observed after CSF1R inhibition on the systemic compartment. However, we observed a reduction in microglial proliferation after IL-34 inhibition in prion-diseased mice, indicating that microglia could be more specifically targeted by reducing IL-34. Overall, our results highlight the challenges of targeting the CSF1R/IL34 axis in the systemic and central compartments, important for framing any therapeutic effort to tackle microglia/macrophage numbers during brain disease.

Keywords: CSF1R (colony-stimulating factor 1 receptor), prion disease, tissue-resident macrophage, chronic neurodegeneration, proliferation

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INTRODUCTION

Neuroinflammation is a critical component of neurodegenerative diseases, including Alzheimer's disease (AD), Parkinson's disease (PD), or prion diseases. The neuroinflammatory process is characterized by a robust activation of the innate immune system, with an increase in the number of microglial cells associated with an activated and phagocytic phenotype (1–3). Experimental models of prion disease present several shared features of neurodegenerative diseases including protein misfolding, progressive synaptic degeneration followed by loss of neurons, microglial activation, and production of inflammatory cytokines and chemokines (4).

The contribution of local proliferation of microglia, regulated by the activation of the colony stimulating factor 1 receptor (CSF1R), has been shown as a disease-modifying mechanism during the progression of the ME7 prion model of progressive chronic neurodegeneration (5). Similarly, a prolonged inhibition of the tyrosine kinase activity of CSF1R blocks microglial proliferation and prevents synaptic degeneration, ameliorating disease progression, in the APP/PS1 model (6), the 3xTg model (7), and the 5xFAD model (8, 9) of AD-like pathology. More recently, our group has validated this disease-modifying mechanism in the P301S mouse of tauopathy, demonstrating that inhibition of CSF1R is effective in repolarising microglia to a homeostatic phenotype, preventing neuronal degeneration (10). In recent years, the therapeutic potential of blocking antibodies and small molecule CSF1R kinase inhibitors has been demonstrated in inflammatory diseases, neurological disorders, bone diseases, and cancer, with some candidates currently in clinical phase testing (11–13). However, the broader impact of this approach on the innate immune system remains unclear. CSF1R is expressed by cells of the myeloid lineage (14) and therefore it is anticipated that the inhibition of CSF1R would not only affect microglia, but also have potential on-target effects in circulating and tissue-resident myeloid populations, with a possible downstream immunosuppressive effect. CSF1R can be activated by two independent ligands with high affinity, the colony stimulating factor 1 (CSF-1) (15) and the recently identified interleukin 34 (IL-34) (16). A potential avenue to block this pathway more selectively is the specific modulation of the binding of its ligands, to increase tissue specificity and reduce side effects. Both ligands have been shown to promote microglial proliferation (5) but also show a differential temporal and spatial pattern of expression. Whereas CSF-1 is broadly expressed (spleen, lymph nodes, cortex, bone marrow, amongst others), the expression of IL-34 is restricted to a few tissues, predominantly produced in the skin and the brain, with minimal overlap with the CSF-1 expression pattern (17, 18). Interestingly, mice lacking IL-34 ($Il34^{LacZ}$) displayed a marked reduction of Langerhans cells in the skin and microglial cells in the brain, whereas other tissue macrophages were unaffected, showing that IL-34 specifically controls the development and maintenance of these populations (19, 20).

Since IL-34 has been shown to be a tissue-restricted ligand of CSF1R, and it is crucial for the differentiation and maintenance of microglial cells in the brain, we aimed to investigate whether IL-34 inhibition could be used as a selective approach to reduce microglial proliferation during chronic neurodegeneration with minimal effects on other tissue-resident myeloid populations. Here, we first describe the effects of the selective inhibition of IL-34, compared to CSF1R inhibition, on different tissue-resident populations in healthy mice, supporting tissue-selectivity of IL-34. We also demonstrate that IL-34 inhibition decreases the proliferation of microglial cells in the ME7 prion model, showing that IL-34 is involved in the regulation of microglial proliferation and supporting that the inhibition of this cytokine could be used as a more selective approach to modulate microglial proliferation in neurodegenerative diseases.

METHODS

In vitro Assessment of CSF1R Phosphorylation

The N13 murine microglia cell line (21) was cultured in Dulbecco's modified Eagle's medium (DMEM, Thermo Fisher Scientific), supplemented with 10% fetal bovine serum and 50 U/mL penicillin/ streptomycin (Thermo Fisher Scientific). Cells were maintained in T75 flasks at 37°C in a 5% CO_2 humidified atmosphere. Cells were plated at a density of 2×10^5 cells/cm^2 in 6-well-plates and cultured overnight to allow adherence. Cells were plated at a density of 2×10^5 cells/cm^2 in 6-well-plates and cultured overnight to allow adherence. Cells were kept in serum-free medium for 4 h prior to stimulation and then incubated for the indicated time points (5 or 10 min) with recombinant CSF-1 (50 or 100 ng/mL), IL-34 (50 or 100 ng/mL) (R&D Systems) or LPS (1 μg/mL) as a negative control for CSF1R pathway activation (22, 23), after which cells were immediately lysed in RIPA buffer (Thermo Fisher Scientific), supplemented with protease and phosphatase inhibitor cocktails (Roche, Thermo Fisher Scientific). Protein lysates were concentrated using Microcon-10kDa Centrifugal Filter Units (Merck Millipore), according to manufacturer's instructions and protein concentration was determined using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). Protein lysates were subjected to SDS-PAGE and Western blot.

In vitro Assessment of IL-34 Neutralizing Antibodies Using CellTiter Glo

Mouse myelogenous leukemia (M-NFS-60) cells were CSF-1 (R&D systems, 216-MC/CF) starved for 24 h. In white clear bottom 96-well-plates 10 μL IL-34 antibody (mouse monoclonal IgG2A (v1.1 manufactured by Genscript, (24, 25)), rat monoclonal IgG2A (MAB5195, R&D Systems) and sheep polyclonal IgG (AF5195, R&D Systems) and 10 μL IL-34 stimulus (R&D systems, 5195-ML-CF) were incubated at 37°C for 30 min before 80 μL M-NFS-60 cells (10^3 cells/well) were added. After two days incubation at 37°C cell viability was assessed using CellTiterGlo (Promega, G7570) following manufacturer's instructions. Hundred microliter reconstituted CellTiterGlo was added per well, plates were shaken for 2 min and incubated at room temperature for 10 min before luminescence was read.

Experimental Model of Prion Disease and Pharmacological Treatments

C57BL/6J mice (Harlan laboratories) and *c-fms*-eGFP (macgreen) mice (26), characterized by eGFP expression under the *c-fms* (CSF1R) promoter, were bred and maintained in local facilities. Mice were housed in groups of 4–10, under a 12 h light/12 h dark cycle at 21°C, with food and water *ad libitum*. To induce prion disease 6 weeks old C57BL/6J or macgreen mice were anesthetized with a ketamine/xylazine mixture (85 and 13 mg/kg), and 1 μL of ME7-derived (ME7 group) brain homogenate (10% w/v) was injected stereotaxically and bilaterally at the dorsal hippocampus, coordinates from bregma: anteroposterior, −2.0 mm; lateral, ±1.7 mm; depth, −1.6 mm. Naïve animals were used as controls. All procedures were performed in accordance with U.K. Home Office licensing. Group sizes were defined after performing power calculations, in order to achieve a significant difference of $p < 0.05$, in light of a retrospective analysis of our previous published results, to reach a power between 0.80 and 0.90, depending on the specific experimental conditions. The effect size was calculated taking into consideration the strength of association between the variables, the sensitivity, and the variation of any dependent variable. The calculations are the customary ones based on normal distributions.

For chronic treatment of healthy mice, rat monoclonal CSF1R-blocking antibodies (BE0213, Bio X Cell) and rat monoclonal IL-34 antibodies (MAB5195, R&D Systems) were diluted in PBS, pH 7.4 and administered by intraperitoneal injections 3 times a week for 3 weeks at a dose of 250 μg per injection. For chronic treatment in ME7 prion mice, mouse monoclonal IL-34 IgG2A (24) was administered biweekly for 4 weeks at a dose of 60 mg/kg, starting 12 weeks after prion inoculation. For acute treatment in ME7 prion mice, 1 μg of mouse or human-specific IL-34 neutralizing antibody (sheep polyclonal IgG, AF5195, or AF5265, R&D Systems) was stereotaxically and bilaterally injected into the dorsal hippocampus, coordinates from bregma: anteroposterior, ?2.0 mm; lateral, ±1.7 mm; depth, ?1.6 mm, 12 weeks after induction of prion disease. Mice received daily intraperitoneal BrdU injections (7.5 mg/mL, 0.1 mL/10 g weight in sterile saline; Sigma-Aldrich) and were sacrificed 1 week after intracerebral antibody administration. Weight of the mice was monitored in all experiments, and no differences were observed between treated and untreated groups. All the experimental groups were randomized to avoid gender and cage effects, and all the experiments were performed double-blinded.

Histology

Mice were terminally anesthetized with an overdose of sodium pentobarbital and transcardially perfused with heparinised 0.9% saline. Brain and peripheral organs (liver, kidney, and spleen) were fixed in 4% paraformaldehyde overnight and immersed in 30% sucrose in PBS for at least 24 h. Tissue was cut in serial sections (35 μm thick, coronal sections of the brain) with a cryostat (Leica) and stored free-floating in cryoprotectant solution [30% sucrose, 30% ethylene glycol, 1% Polyvinyl-pyrrolidone (PVP-40) in 0.1 M PB, pH 7.4] at −20°C. For histological analysis of peripheral organs, 4–6 sections from each organ were randomly selected. For analysis of cortex, hippocampal CA1 and dentate gyrus, every 6th systematically sampled section, spanning the entire area of the hippocampus and totalling 6–9 sections were used for quantification. Tissue sections taken from macgreen mice were directly mounted on slides with Mowiol/DABCO (Sigma-Aldrich) mixture. Immunohistochemistry of brain sections from C57BL/6J mice was performed as previously described (5). Briefly, sections were subjected to DNA denaturation with 2N HCl (30 min, 37°C), followed by incubation with 5% normal serum and 5% BSA in PBS to block non-specific binding. After rinses with PBS-0.1% Tween 20 (PBST), sections were incubated overnight at 4°C with rabbit anti-Iba1 (Wako, 019-19741) and anti-BrdU (Biorad, MCA2060). After washes with PBST, sections were incubated with host-specific Alexa 488- and 568-conjugated secondary antibodies (Invitrogen). Brain sections and sections of peripheral organs from macgreen mice mounted with Mowiol/DABCO were imaged with a Leica DM5000B microscope coupled to a Leica DFC300FX camera. Iba1-positive, BrdU/Iba1 double positive and eGFP-positive cells in brain and organs of macgreen mice were counted using the cell counter tool of the ImageJ software and cell number was normalized to the quantified area.

Analysis of Skin-Resident Langerhans Cells

Ears were fixed in 4% paraformaldehyde overnight and then stored in PBS. The ears were split into dorsal and ventral halves and each pair was mounted on slide under coverslip mounted in Vectashield anti-fade mounting medium. For each half ear, 5 fields were chosen at random and 0.9-μm thick sections were collected on a LSM700 confocal microscope (Zeiss) using settings for eGFP fluorescence with a 40 × objective. For each field a z-stack was taken to cover the full thickness of the Langerhans cells layer-typically 5–100 images depending on the flatness of the ear. Cell volume and number were measured using Volocity software (Quorum Technologies). Cells were identified as those with a GFP intensity >2SD from the mean image intensity. Non-cellular objects <200 or >5,000 μm^3 were excluded. Objects with a long axis >100 μm were also excluded-this eliminated most auto-fluorescent hairs in the z stacks. Each image was checked manually to remove cell doublets and unexcluded hair profiles.

Flow Cytometric Analysis of Brain and Blood Samples

Mice were terminally anesthetized with an overdose of sodium pentobarbital and transcardially perfused with heparinised 0.9% saline. Brain hemispheres were harvested in PBS with 2% FCS and 2 mM EDTA (FACS buffer) and mechanically triturated and enzymatically dissociated using the Neural Tissue Dissociation Kit (P) (Miltenyi), according to manufacturer's instructions. Samples were passed through a cell strainer of 70 μm mesh (BD2 Falcon) with FACS buffer, and centrifuged at 500 × g for 10 min at 4°C. After the second wash, cells were re-suspended in 37%

Percoll (GE Healthcare) and centrifuged at 500 × g for 30 min at 18°C. The supernatant and myelin layers were discarded, and the cell pellet enriched with mononuclear cells was resuspended in FACS buffer. Blood samples were harvested by cardiac puncture and collected in EDTA-coated tubes. Brain cells and blood samples were split in several tubes and immunostained. Primary antibody labeling was performed for 1 h at 4°C, using the following antibodies: rat-anti-mouse CD11b (BD Biosciences, clone M1/70), rat-anti-mouse CD45 (Biolegend, clone 30-F11) and rat-anti-mouse Ly6C (BD Biosciences, clone AL-21) and Fixable Viability Dye eFluor™ 450 (eBioscience). Moreover, unstained cells and isotype-matched control samples were used to control for autofluorescence and non-specific binding of antibodies. After staining, erythrocytes in blood samples were lysed in RBC lysis buffer (eBioscience). Cells were washed and resuspended in FACS buffer. Samples were run on a BD FACS Aria Flow cytometer, recording 100,000 events per sample. Data was analyzed using FlowJo software.

SDS-PAGE and Western Blot

Frozen brain samples and peripheral organs were homogenized in T-PER™ Tissue Protein Extraction Reagent (Thermo Fisher), N13 cells were lysed in RIPA buffer (Thermo Scientific), supplemented with protease inhibitors (EASYpack, Roche) and phosphatase inhibitors (PhosSTOP, Roche). Homogenates were centrifuged at 13,000 rpm and the supernatant was collected. Protein was quantified using BCA assay (Thermo Fisher) following the manufacturer's instructions.

40 μg protein of N13 cell lysates was loaded to 7.5% Mini-PROTEAN® TGX Stain-Free™ Protein Gels (BioRad) and transferred to nitrocellulose membrane using the Trans-Blot® Turbo™ RTA Mini Transfer Kit (BioRad). After blocking with 5% BSA in TBS/0.1% Tween20, membranes were incubated with a combination of rabbit polyclonal antibodies against phospho-M-CSF receptor (Tyr546, Tyr708, Tyr723, and Tyr923, Cell signaling), phospho-AKT (Ser473, Cell signaling), or phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204, Cell signaling) over night at 4°C. Membranes were washed in TBS and further incubated with an HRP-labeled anti-rabbit IgG (BioRad) for 2 h at room temperature. Membranes were incubated with the SuperSignal™ West Pico Chemiluminescent Substrate (Thermo Fisher Scientific) and signal was detected on the ChemiDoc Imaging System (BioRad). Membranes were stripped using the Restore™ Western Blot Stripping Buffer (Thermo Fisher Scientific) and reprobed with mouse monoclonal CSF-1R antibody (D-8, Santa Cruz Biotechnology), anti-AKT (Cell signaling), or anti-ERK1/ERK2 antibody (9B3, abcam), followed by HRP-labeled anti-mouse or anti-rabbit IgG antibody (BioRad). Intensity of protein bands was quantified using Adobe Photoshop.

Elisa

Nunc MaxiSorp™ flat-bottom 96-well-plates (Thermo Scientific) were coated with F(ab')2 fragment anti-rat or anti-mouse IgG (H+L) (Jackson ImmunoResearch) overnight. Plates were washed with PBS + 0.05% Tween20 and incubated with blocking buffer (PBS containing 0.05% Tween20 and 1% BSA) to block non-specific binding sites. Plasma samples or tissue lysates diluted in blocking buffer were incubated for 2 h or overnight. A standard was generated using respective anti-IL-34 or anti-CSF1R antibodies that were used for *in vivo* treatment. After washing, plates were incubated with peroxidase-conjugated anti-rat or anti-mouse Fcγ subclass 2a-specific IgG (Jackson ImmunoResearch) for 2 h, then washed and incubated with 1-Step™ Ultra TMB-ELISA Substrate Solution (Thermo Scientific). The reaction was stopped with 2N H_2SO_4 and the signal was measured on a plate reader at 450 nm.

CSF-1 and IL-34 in plasma or brain were measured by commercially available immunoassays (R&D systems), according to manufacturer's instructions.

Statistics

Data are shown as mean ± SEM and were analyzed using the GraphPad Prism 6 software package (GraphPad Software), using two-way ANOVA with Tukey's *post-hoc* test for multiple comparisons, Student's *t*-test or one-way ANOVA followed by Tukey's *post-hoc* test for multiple comparisons, as indicated. Differences were considered significant at $p < 0.05$.

RESULTS

IL-34 Induces Activation of the CSF1R Signaling Pathway and IL-34-Mediated Cell Growth Can Be Inhibited Using Neutralizing Antibodies

To investigate whether IL-34 activates the CSF1R pathway in microglia, we stimulated a murine microglia cell line (N13) with either IL-34 or with CSF-1 and analyzed tyrosine phosphorylation of the receptor and downstream signaling molecules ERK1/ERK2 and AKT. Stimulation with either IL-34 or CSF-1 leads to increased phosphorylation of CSF1R and downstream mediators, indicating that IL-34 binds to and activates the CSF1R pathway, triggering downstream signaling pathways related to survival and proliferation (**Figure 1A**). IL-34-mediated growth of myelogenous leukemia cell line M-NFS-60 can be inhibited by three different IL-34 neutralizing antibodies, which were further used in this study and showed similar potencies [mouse monoclonal v1.1 (25): IC_{50} 0.43 nM, rat monoclonal MAB5195: IC_{50} 0.53 nM, sheep polyclonal AF5195: IC_{50} 2.05 Nm] (**Figure 1B**), indicating that CSF1R-dependent signaling can be modulated by IL-34 inhibition.

CSF1R- but Not IL-34 Antibody-Mediated Inhibition Leads to a Reduction of $CSF1R^+/Ly6C^{lo}$ Blood Monocytes

To determine the effect of CSF1R *vs.* IL-34 blockade on blood immune cells, macgreen mice were treated by intraperitoneal injections of CSF1R- or IL-34- neutralizing antibodies (monoclonal rat IgG2a, 250 μg per injection, 3 injections per week), vehicle (PBS), or rat IgG2a isotype for 3 weeks (**Figure 2A**). The use of macgreen mice allows to monitor the abundance of $CSF1R^+$ cells based on the *csf1r*-eGFP transgene reporter expression. Measuring antibody titers in the plasma

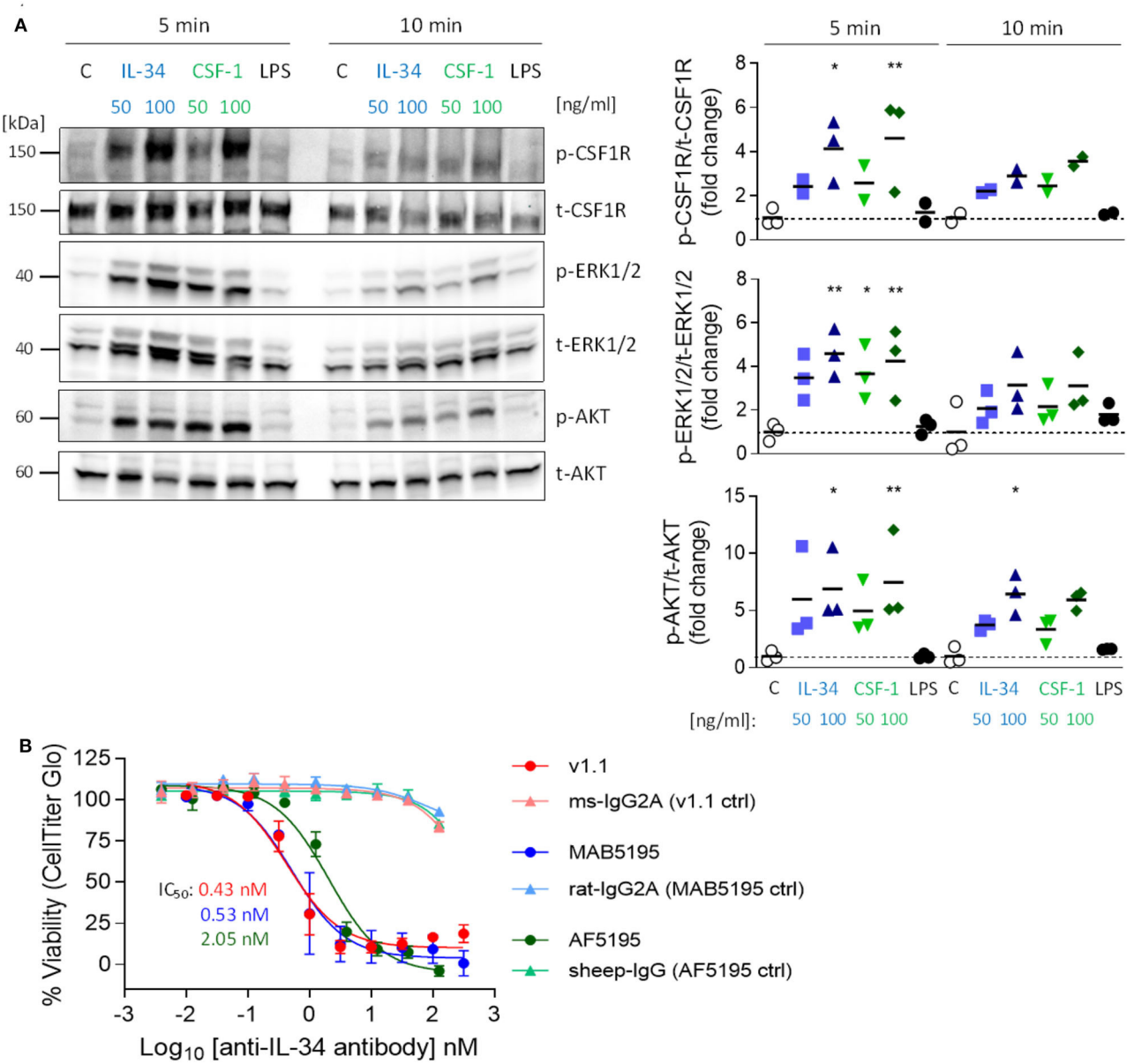

FIGURE 1 | Activation of the CSF1R signaling pathway by IL-34 and CSF-1 and inhibition of IL-34-mediated cell growth using IL-34 neutralizing antibodies. **(A)** N13 microglia cell line was stimulated with IL-34 (50 or 100 ng/mL), CSF-1 (50 or 100 ng/mL) or LPS (1 μg/mL) for 5 or 10 min. Cell lysates were subjected to Western blotting which showed increased phosphorylation of CSF1R and downstream ERK1/2 and AKT after IL-34 and CSF-1 stimulation. **(B)** IL-34 neutralizing antibodies used in this study (mouse monoclonal IgG2A [v1.1, (24)), rat monoclonal IgG2A (MAB5195, R&D Systems) and sheep polyclonal IgG (AF5195, R&D Systems)] prevented IL-34-dependent cell growth of M-NFS-60 in a similar nanomolar range. $n = 3$, data shown represent mean ± SEM, two-way ANOVA followed by Tukey's multiple comparison test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, comparisons are stimulations vs. unstimulated control **(C)** for each time point.

following treatment using a rat IgG2a-specific ELISA showed comparable levels of antibody in anti-IL-34 and anti-CSF1R treated mice, while levels of isotype control were found to be significantly higher (**Figure 2B**). Administration of CSF1R antibodies, but not IL-34 antibodies, increased CSF-1 levels in the plasma (**Figure 2C**), which has been described as an indication of target engagement (27). Levels of IL-34 were found to be low in the plasma at baseline (~30 times lower than CSF-1 levels) and were increased after CSF1R antibody treatment similar to CSF-1 levels, while IL-34 was undetectable after IL-34 antibody treatment (**Figure 2D**). Flow cytometric analysis of blood immune cells demonstrated a significant decrease in $CSF1R^{+}$ monocytes after CSF1R antibody treatment, which, although a slight trend toward a reduction in cell number can be

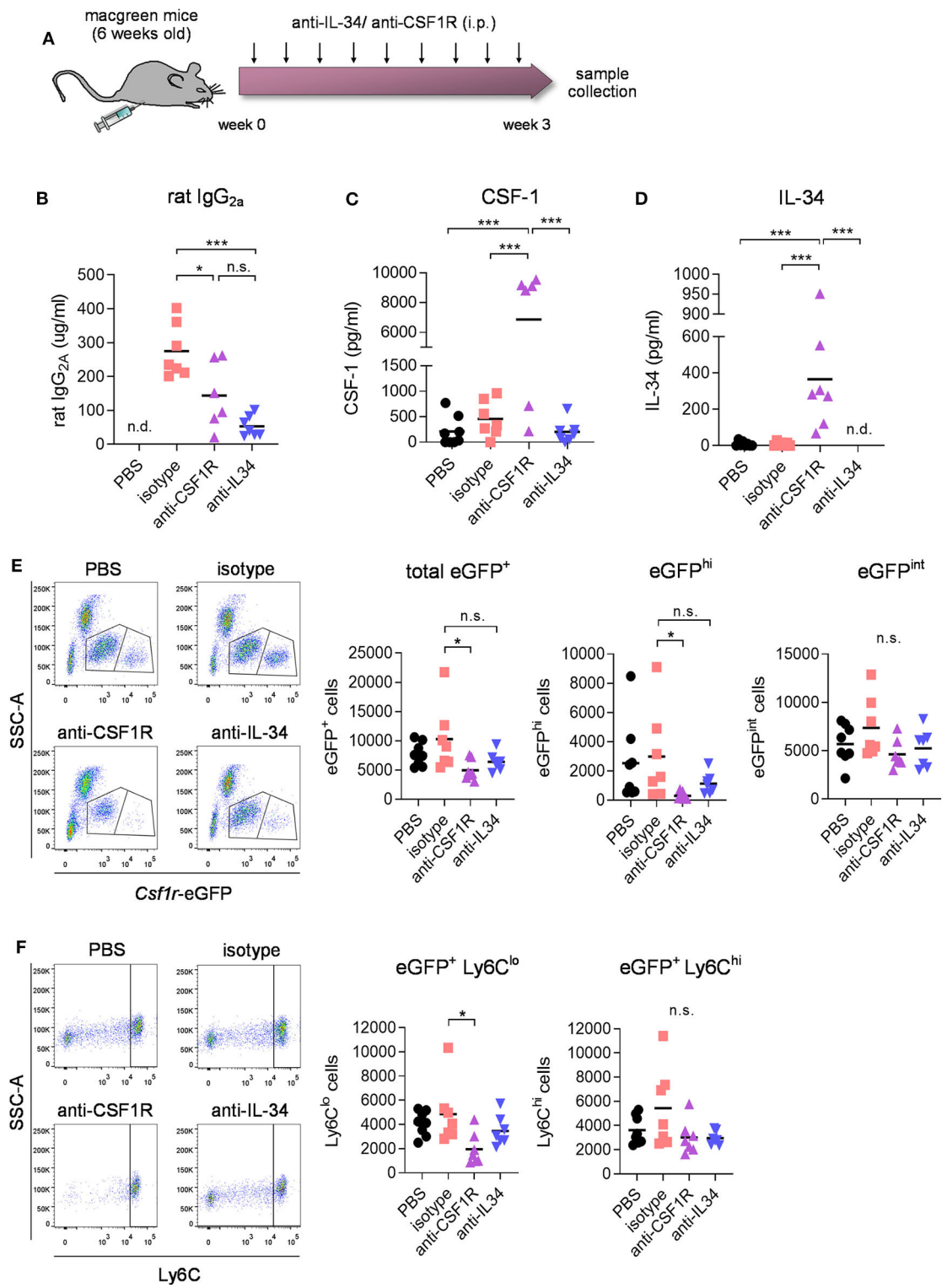

FIGURE 2 | Effect of CSF1R- and IL-34 antibody treatment on blood immune cell compartment. **(A)** Macgreen mice were treated with anti-CSF1R or anti-IL-34 (both rat monoclonal IgG2A) by intraperitoneal injections of 250 μg antibody 3x a week for 3 weeks. **(B–D)** Levels of rat IgG2a, CSF-1 and IL-34 were measured in blood plasma after the treatment by ELISA, which showed increased IL-34 and CSF-1 levels after CSF1R- but not IL-34 blockade. **(E)** Flow cytometric analysis of CSF1R$^+$ cells in the blood of anti-CSF1R- and anti-IL-34- treated mice demonstrated a significant reduction after CSF1R- but not IL-34 antibody treatment. Graphs indicate

(Continued)

FIGURE 2 | respective cell numbers per 5×10^4 $CD45^+$ cells. **(F)** Flow cytometry of $eGFP^+$ $Ly6C^{hi}$ and $eGFP^+$ $Ly6C^{lo}$ subpopulations of CSF1R-expressing cells in the blood shows a reduction in $Ly6C^{lo}$ cells after anti-CSF1R treatment, while $Ly6C^{hi}$ cells were not affected. Graphs indicate respective cell numbers per 5×10^4 $CD45^+$ cells. PBS $n = 8$, isotype $n = 8$, anti-CSF1R $n = 8$, anti-IL-34 $n = 7$, data shown represent mean ± SEM, two-way ANOVA followed by Tukey's multiple comparison test. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

observed, was not significant after IL-34 antibody administration (**Figure 2E**, for gating strategy see **Supplementary Figure 1**). Further analysis of $CSF1R^+$ subpopulations showed that the reduction in blood monocytes after CSF1R antibody treatment is largely due to an effect on cells with high CSF1R expression ($eGFP^{hi}$) as opposed to cells with intermediate CSF1R expression ($eGFP^{int}$, **Figure 2E**). The anti-CSF1R-sensitive $eGFP^{hi}$ cells have been previously identified as $CD14^+$ $CD16^{++}$ human non-classical monocytes, while the $eGFP^{int}$ population constitutes the $CD14^{++}$ $CD16^{+/-}$ intermediate and classical monocytes (28), equivalent to the murine non-classical $Ly6C^{lo}$ monocytes and the classical $Ly6C^{hi}$ monocytes, respectively. In accordance with an effect on $eGFP^{hi}$ cells, the non-classical $Ly6C^{lo}$ monocytes were predominantly reduced after anti-CSF1R treatment, while classical $Ly6C^{hi}$ were not affected (**Figure 2F**). Again, both $Ly6C^{hi}$ and $Ly6C^{lo}$ populations were not significantly reduced after IL-34 antibody administration. In contrast to blood immune cells, $CSF1R^+$ cells in the bone marrow were not affected by either CSF1R or IL-34 antibody treatment, indicating that CSF1R is not required for the differentiation of myeloid progenitors in the marrow (**Supplementary Figures 2A,B**). Taken together, these results indicate that the number of $CSF1R^+$ monocytes in the blood depends on CSF1R, and is less dependent on IL-34.

Systemic IL-34 Blockade Reduces Epidermal Langerhans Cells, but Not Macrophage Populations in Liver and Kidney or Microglia in the Brain

We next aimed to determine the effect of IL-34 and CSF1R antibody treatment on different populations of tissue-resident macrophages. Measurement of rat IgG2a levels in liver, kidney, spleen, and brain by ELISA showed equal distribution of IL-34- and CSF1R- neutralizing antibodies in each organ, with a distribution between different organs from highest to lowest as follows: spleen > kidney > liver > brain (**Supplementary Figure 3**). Administration of IL-34 neutralizing antibodies for 3 weeks did not change the number of $CSF1R^+$ macrophages in the liver and in the kidney (**Figure 3A**). In contrast treatment with a CSF1R blocking antibody lead to a pronounced reduction of macrophages in both organs, demonstrating a 41% reduction in liver-resident macrophages and a 85% reduction of macrophages in the kidney (**Figure 3A**). Skin-resident $CSF1R^+$ Langerhans cells, were significantly decreased after treatment with either IL-34- or CSF1R blocking antibodies (**Figure 3A**). This indicates that skin-resident Langerhans cells depend on IL-34- mediated signaling through CSF1R, while macrophages in liver and kidney are maintained by IL-34- independent CSF1R signaling.

To find out whether IL-34 antibody treatment affects microglia number in the brain we quantified the number of $CSF1R^+$ cells in the cerebral cortex, hippocampal CA1, and dentate gyrus. Peripheral administration of CSF1R or IL-34 blocking antibodies for 3 weeks did not overtly affect the number of microglia in the brain, with only a small reduction in the CA1 region of the hippocampus observed after anti-IL-34 administration (**Figure 3B**). Since previous reports showed that CSF1R inhibition using small molecule inhibitors leads to a reduction in microglia (5), it is likely that we did not reach optimal antibody penetration into the brain with the administered dose of antibody to achieve sufficient target engagement.

Chronic Systemic Administration of IL-34 Blocking Antibody Lacks Sufficient Central Target Engagement, Not Modifying the Microglial Population in ME7 Prion Mice

We next aimed to investigate whether chronic systemic IL-34 antibody treatment would affect microglia numbers in the ME7 prion mice, a model of neurodegeneration which is characterized by a pronounced expansion of the microglia population (5). Based on our previous observation showing a lack of effect in the brain with 250 μg antibody per injection (∼10 mg/kg, **Figure 3B**), we increased the administered dose to 60 mg/kg per injection (**Figure 4A**). After 4 weeks of biweekly intraperitoneal injections of the antibody in prion diseased mice, microglia populations were analyzed by flow cytometry and histology. While ME7 prion mice showed increased numbers of microglia compared to naïve animals, as assessed by flow cytometry ($CD45^{int}$ $CD11b^+$ cells) and histology (*Csf1r*-$eGFP^+$ cells), there was no difference in microglia numbers in brains of anti-IL-34 treated animals compared to control-treatment (**Figures 4B,C**). The proportion of $CD45^{hi}$ $CD11b^+$ cells was not affected by disease or treatment, indicating no effect on infiltration of peripheral myeloid cells into the brain (**Figure 4B**). Levels of isotype and anti-IL-34 antibody were detectable in plasma and brain using a mouse-IgG2a specific ELISA, which revealed higher levels of isotype compared to IL-34 antibody in both compartments, with a brain/plasma ratio of 0.165 for the isotype and 0.141 for the IL-34 antibody (**Figure 4D**). CSF-1 levels in the brain were significantly increased in prion mice compared to naïve mice, but unaffected by the anti-IL34 treatment (**Figure 4E**). IL-34 levels were around 300 times higher in the naïve brain than CSF-1 levels, but not changed in the context of prion disease or after IL-34 antibody treatment (**Figure 4E**). In order to determine whether the absence of an effect of IL-34 antibody treatment on microglia numbers could be due to insufficient target engagement, we developed an ELISA to capture mouse IgG2a from brain lysates, followed by detection of IL-34 molecules bound to captured IgG2a. Using this assay we detected bound IL-34 exclusively in brain

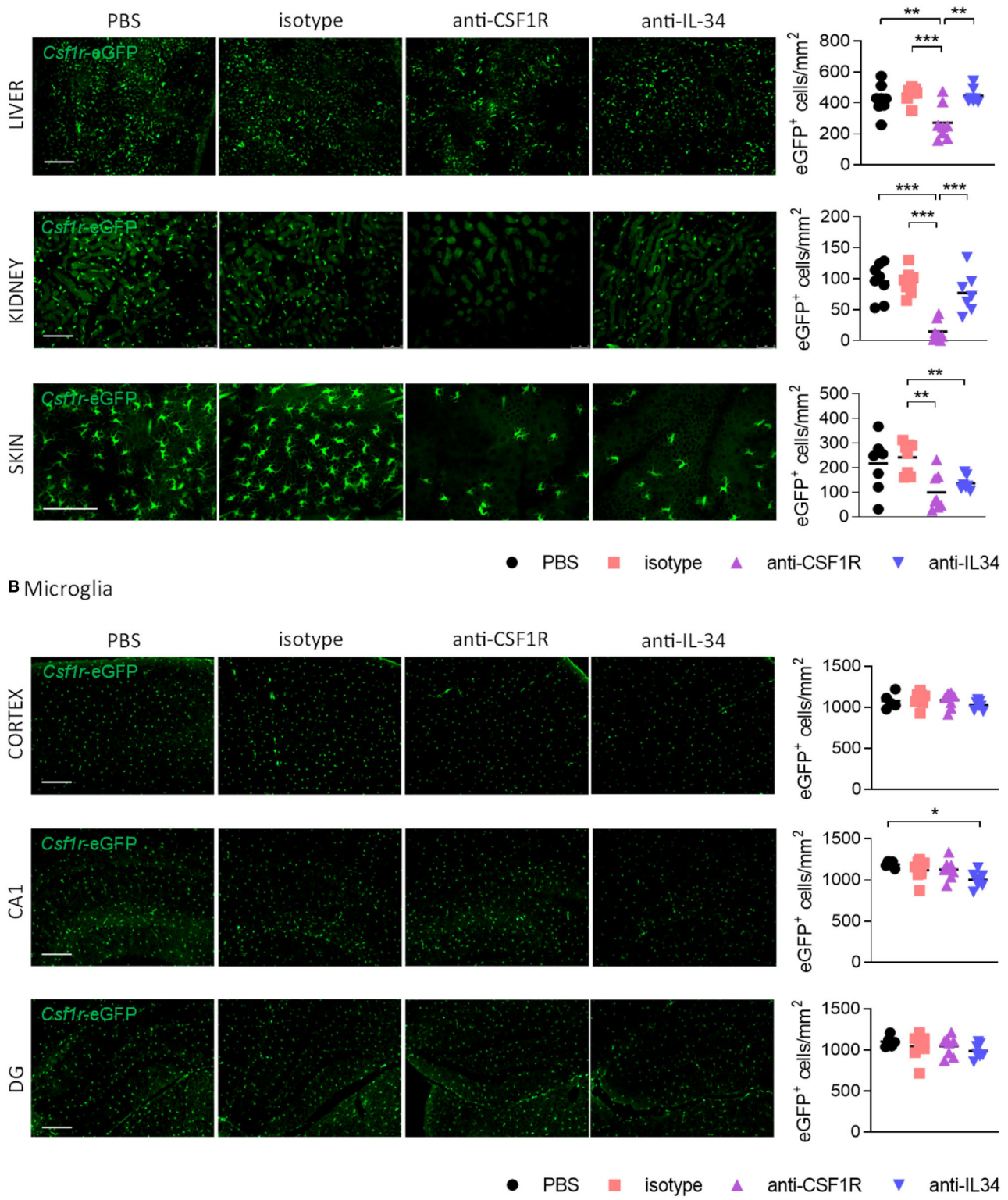

FIGURE 3 | Effect of CSF1R- and IL-34 antibody treatment on tissue macrophages. **(A)** Histological analysis of CSF1R-positive cells in liver and kidney shows a reduction after anti-CSF1R treatment, but not after anti-IL-34 treatment. Skin Langerhans cells were significantly reduced after both CSF1R – and IL-34 blockade. **(B)** Microglia in cortex, hippocampal CA1 and dentate gyrus were not majorly affected by CSF1R or IL-34 antibody administration. Scale bar 100 μm. PBS $n = 8$, isotype $n = 8$, anti-CSF1R $n = 8$, anti-IL-34 $n = 7$, data shown represent mean ± SEM, two-way ANOVA followed by Tukey's multiple comparison test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

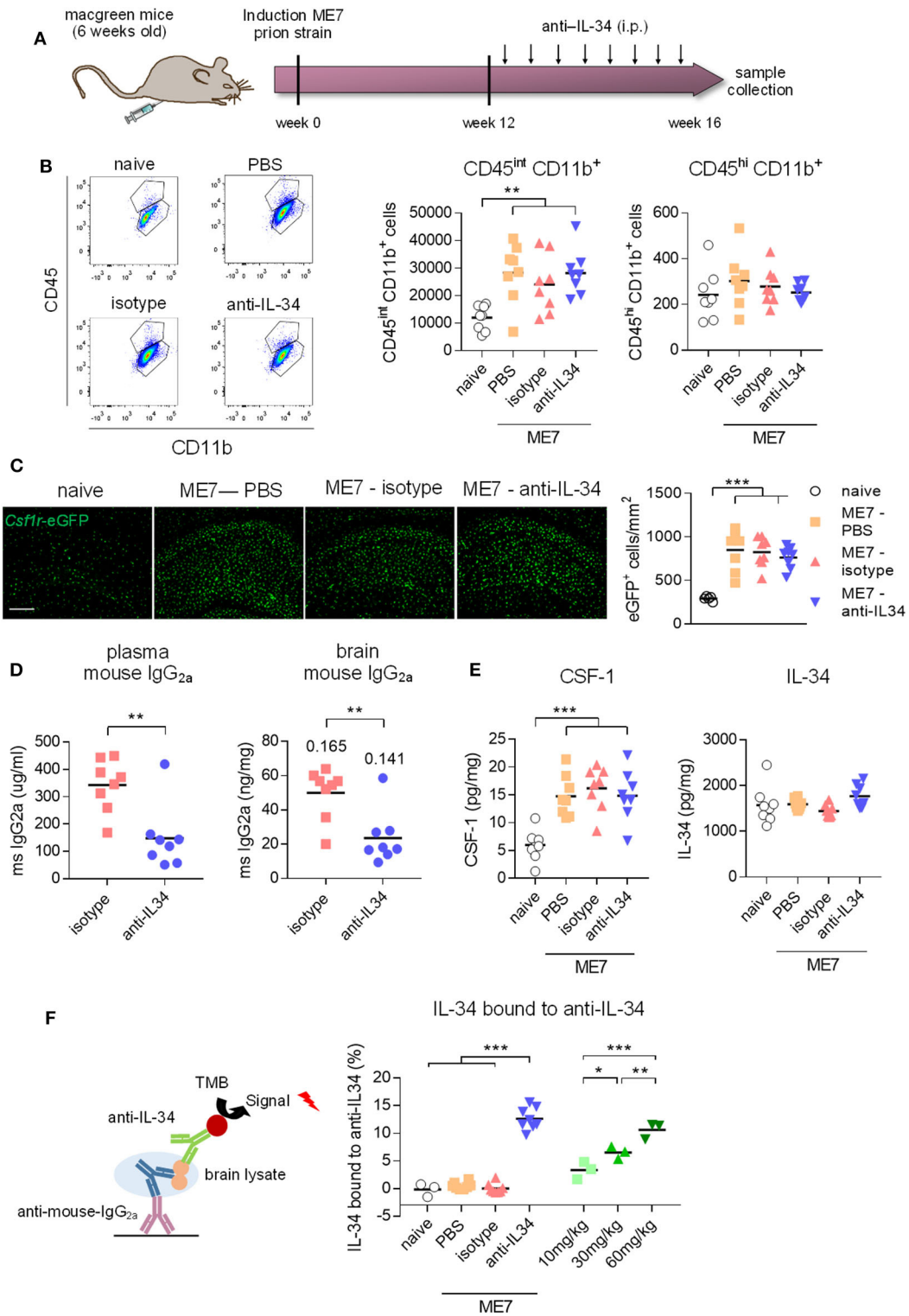

FIGURE 4 | Peripheral IL-34 antibody injections in ME7 prion mice did not affect microglia numbers. **(A)** Macgreen mice infected with prion disease were treated with anti-IL-34 (mouse monoclonal IgG2A) by intraperitoneal injections at a dose of 60 mg/kg twice a week for 4 weeks. **(B)** Flow cytometric analysis of CSF1R-positive cells in the brain of anti-IL-34- treated mice showed no effect on number of $CD45^{int}$ $CD11b^{+}$ or $CD45^{hi}$ $CD11b^{+}$ cells. Graphs indicate respective cell numbers per 10^5 living cells. **(C)** Histological analysis of CSF1R-positive cells in the cortical brain shows unchanged microglial numbers after anti-IL-34 treatment. **(D)** Levels of mouse IgG2a were measured in blood plasma and brain lysates by ELISA, which showed higher levels of isotype than anti-IL-34. Values for tissue/plasma ratio are indicated above bars. **(E)** CSF-1 and IL-34 were measured in brain lysates, showing no alterations of IL-34 and CSF-1 levels after anti-IL-34 administration.

(Continued)

FIGURE 4 | (F) IL-34 bound to anti-IL34 in brain lysates were detected by ELISA, by coating plates with anti-mouse IgG2a to capture IL-34 antibodies present in brain lysates and detecting IL-34 bound to IL-34 antibodies with an IL-34 specific detection antibody (R&D systems). Levels of IL-34 bound to IL-34 antibodies were <15% from total IL-34 levels. Scale bar 100 μm. $n = 8$ per group, data shown represent mean ± SEM, two-way ANOVA followed by Tukey's multiple comparison test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

lysates of mice treated with IL-34 antibody, but not with isotype or PBS (**Figure 4F**). The percentage of IL-34 bound to IL-34 antibody showed a dose dependent increase after 2 injections in healthy mice, demonstrating increased target engagement with increasing doses of IL-34 antibody (**Figure 4F**). However, ME7 prion mice treated for 4 weeks with biweekly injections of 60 mg/kg showed that only ∼13% of total IL-34 was bound to the antibody, suggesting a low degree of target engagement (**Figure 4F**).

Intracerebral Administration of IL-34 Blocking Antibodies Reduces Microglia Proliferation in ME7 Prion Mice

Since we did not achieve a significant degree of target engagement in the brain with peripheral antibody administration, we aimed to determine whether administering the antibody directly into the brain of prion mice could affect microglia numbers. IL-34 antibodies were stereotactically injected into the hippocampus 12 weeks after induction of prion disease, a timepoint of pronounced microglia proliferation (5), and brains were collected 1 week after injection (**Figure 5A**). Analysis of cells double-positive for the proliferation marker BrdU and Iba1, a marker of microglia/macrophages, showed increased microglial proliferation and increased total number of Iba1-positive cells in the hippocampus of prion mice compared to naïve mice, while injection of a mouse-specific IL-34 neutralizing antibody significantly reduced microglia proliferation by about 50% (**Figure 5B**). Administration of a human-specific IL-34 antibody did not have an effect on microglia proliferation, possibly due to reduced homology with mouse IL-34 [71% (16)]. Microglial proliferation was higher in ME7 compared to ME7 mice treated with isotype, probably due to the injection itself causing microgliosis associated to the local injury, which naïve and untreated ME7 mice did not receive. The reduction of microglial proliferation however did not result in a reduction of total microglia numbers after IL-34 antibody administration at the analyzed timepoint, probably due to the acute and transient nature of the intervention, which merely affected the small proliferating sub-population of the total microglial population (**Figure 5B**). The finding that microglia proliferation was locally reduced after direct intracerebral injection provides proof-of-concept that IL-34 is involved in regulating microglia proliferation in the context of chronic neurodegeneration and that IL-34 blockade could be used as a strategy to reduce microglia proliferation.

DISCUSSION

In this study we explored the effect of inhibition of IL-34 on blood monocytes, systemic tissue macrophages, and microglia in health and neurodegenerative disease, modeled by a murine model of prion disease. IL-34 is a tissue-specific ligand of the CSF-1 receptor predominantly expressed in the brain and in the skin and has been shown to be crucial for the development and survival of microglia and epidermal Langerhans cells (19, 20). We showed here that inhibition of IL-34 does not affect monocytes and macrophage populations in many peripheral tissues, with an exception of skin-resident Langerhans cells. Even though we did not achieve sufficient target engagement in the brain after peripheral administration of IL-34 blocking antibodies, we observed a local reduction of microglia proliferation when injecting IL-34 antibodies directly into the brain of mice infected with prion disease, indicating that IL-34 is involved in driving proliferation of microglia in the context of neurodegenerative disease.

IL-34 and CSF-1 were shown to activate the CSF1R signaling cascade in a similar manner (29) and have an overall similar effect on human monocyte differentiation *in vitro* as shown by transcriptional profiling and pathway analysis (30). We have also found that the activation pattern of CSF1R and downstream pathway components AKT and ERK1/2 induced by IL-34 resembles the one induced by CSF-1 in microglia. Although IL-34 and CSF-1 seem to similarly affect CSF1R activation and macrophage differentiation, they have distinct tissue expression pattern with limited spatial overlap (29). A more recent report confirms the distinct spatial distribution of IL-34 and CSF-1 in the brain parenchyma, however describes differential transcriptional signatures in microglia exposed to CSF-1 and IL-34, suggesting divergent effects of the two CSF1R ligands on microglia phenotype that might account for the observed differences in regional microglial transcriptional profiles (31). In accordance with previous reports which showed that IL-34 is more widely expressed in the brain than CSF-1 (17, 19, 20), we observed that overall levels of IL-34 protein are ∼300 times higher than CSF-1, highlighting its predominant role in the brain. However, we also observed that IL-34 levels did not further increase in the brain of prion-diseased mice.

It was demonstrated previously that CSF1R inhibition using tyrosine kinase inhibitors can be used as a strategy to decrease microglia proliferation in neurodegenerative disease models, which led to beneficial effects such as reduced neuronal loss and behavioral deficits in mouse models of prion disease (5), tau pathology (10), and Aβ pathology (7, 9). Long-term CSF1R inhibition could potentially increase risk of infections and lead to disturbance of tissue homeostasis due to the reduction of CSF1R-dependent macrophage populations in multiple organs. In a

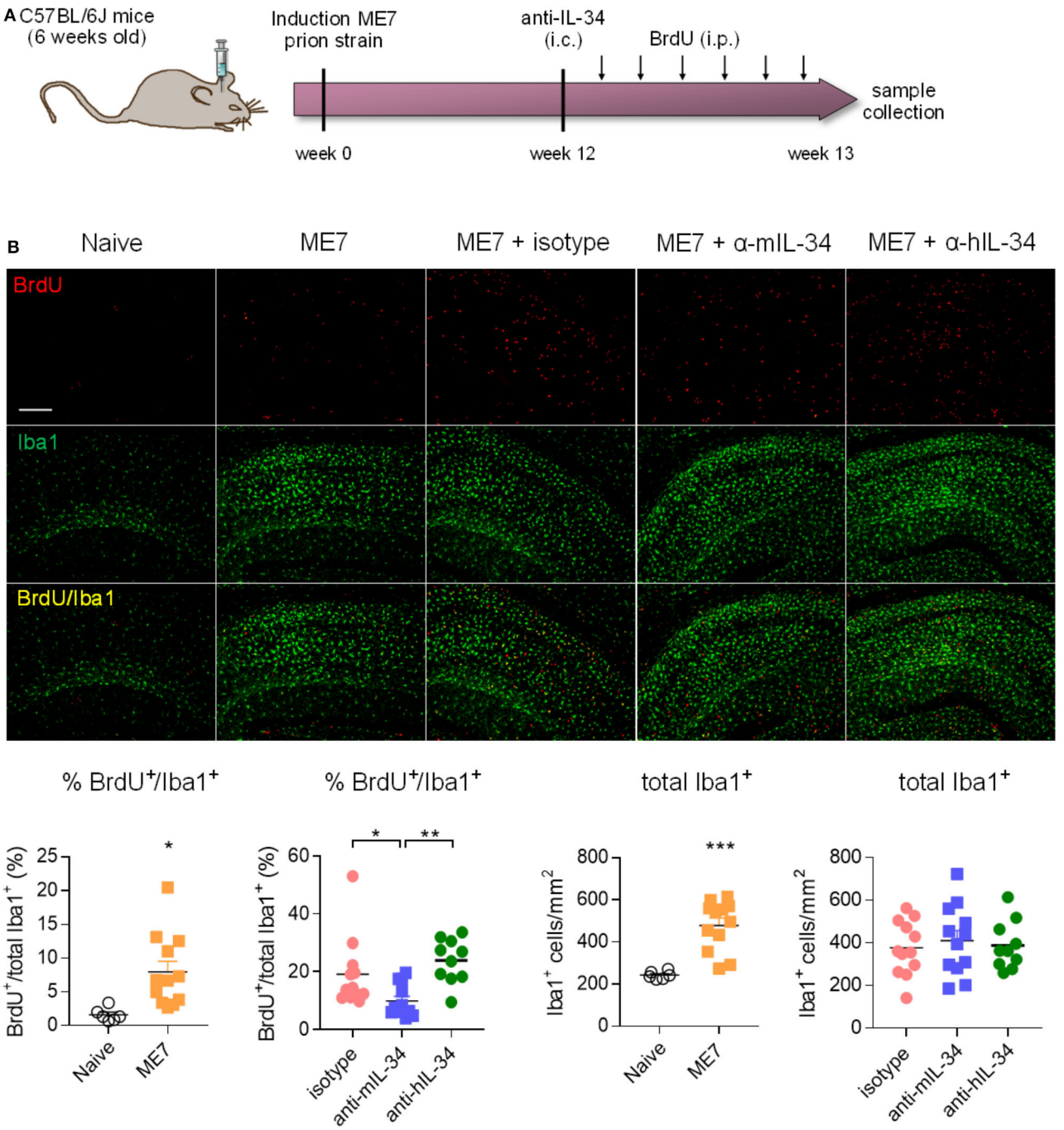

FIGURE 5 | Intracerebral IL-34 antibody administration resulted in reduced microglia proliferation in ME7 prion mice. **(A)** Mice infected with prion disease received a single intracerebral injection of mouse- or human-specific anti-IL-34 (sheep polyclonal IgG) and brains were analyzed 1 week later. **(B)** Histological analysis of BrdU/Iba1-positive microglial cells in the cortex showed a reduction after treatment with a mouse-, but not with a human-specific antibody. Scale bar 100 μm. Naïve $n = 6$, ME7 $n = 12$, ME7 + isotype $n = 10$, ME7 + anti-mIL-34 $n = 8$, ME7 + anti-hIL-34 $n = 7$, data shown represent mean ± SEM, two-way ANOVA followed by Tukey's multiple comparison test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Listeria monocytogenes infection model, CSF-1/IL-34 blockade or treatment with anti-CSF-1 alone increased susceptibility to bacterial infection, although to a lower degree than anti-TNF therapy (32). Interestingly, IL-34 blockade did not alter infection susceptibility (32), indicating an immunosuppressive effect as observed with CSF-1 blockade can be prevented with anti-IL-34 treatment. CSF1R antagonism also lead to higher susceptibility of mice to lethal West Nile virus infection and lack of virologic

control in both brain and periphery, highlighting the importance of myeloid cells in restriction of viral replication and the restimulation of antiviral T cells recruited to the CNS (33). On the other hand, a CSF1R tyrosine kinase inhibitor showed a good safety and tolerability profile in patients with rheumatoid arthritis over a course of 3 months, causing only minor side effects related to compromised Kupffer cell function (27), however the long-term consequences of CSF1R pharmacological inhibition are not well-understood. Other possible side-effects of long-term CSF1R blockade might include disturbances of bone formation and resorption as well as the function of pancreatic β cells (see Martin-Estebane and Gomez-Nicola (34) for further discussion).

A potential way to circumvent affecting multiple macrophage populations is to target IL-34, which is predominantly expressed in the brain. We aimed to evaluate whether inhibition of this more tissue-restricted ligand of CSF1R would cause a reduction in microglia proliferation without having a major impact on peripheral myeloid cell populations. In support of this strategy, the administration of recombinant IL-34 into the brain caused locally increased microglia proliferation similar to CSF-1 (5), showing that IL-34 can induce proliferation of microglia in the brain. In IL-34$^{lacZ/lacZ}$ mice which lack IL-34, microglial numbers are strongly reduced in many brain regions such as cortex, hippocampus and striatum (19, 20), indicating that IL-34 is at least partially responsible for maintenance of the population. Targeting IL-34 using an antibody-based approach, we showed that specific inhibition of IL-34 was sufficient to reduce microglia proliferation present in prion pathology, at least when IL-34 was inhibited over a short period of time by administration of IL-34 antibodies directly into the brain. This finding suggests that IL-34 is partially involved in regulating microglia proliferation in neurodegenerative disease. However, it proved to be challenging in our study to target brain-intrinsic IL-34 using systemically administered neutralizing antibodies probably due to their poor brain penetrance which prevented sufficient antibody titers to efficiently neutralize biological function of IL-34 in the brain. Although two recent reports showed a reduction in microglial numbers after peripheral administration of IL-34-specific monoclonal antibodies at high doses (32, 35), we did not observe a modulation of microglial numbers after chronic systemic antibody treatment in healthy mice or mice infected with prion disease. We found IL-34 to be ∼300 times higher in the brain than CSF-1, while in the blood it was nearly absent. This high abundance of IL-34 in the brain emphasizes the difficulty in efficiently targeting this cytokine with neutralizing antibodies. Similarly, peripheral administration of CSF1R blocking antibodies did not lead to a reduction in microglia in cortex, dentate gyrus and CA1. The fact that blocking CSF1R using small molecule inhibitors resulted in pronounced depletion of microglia in several mouse models (5, 7, 9, 10), further indicates that the strategy applied in this study using blocking antibodies is not favorable. Lin et al. and Easley-Neal et al. provide first proof that manipulation of IL-34 can be used to modify the microglia population in the gray matter of most brain regions (35) and that this approach might be relevant in the context of inflammatory diseases and cancer (32). We extend these findings by showing that in a model of neurodegenerative disease, which is characterized by a pronounced expansion of the microglia population predominantly in the hippocampus, inhibition of IL-34 leads to reduced microglial proliferation. In order to provide further proof-of-concept that IL-34 inhibition can be used to tackle neurodegenerative disease, it is inevitable to apply other strategies which offer an improved brain penetrance profile, ideally by using small molecule inhibitors, which to date does not exist. By contrast, it was recently shown that prion disease induced in IL-34$^{lacZ/lacZ}$ mice did not change the number and activation of microglia and rather accelerated prion disease progression (36). It is possible that in the genetically modified mice with a constitutive loss of function of IL-34, CSF-1 compensates for the absence of IL-34 and drives proliferation through CSF1R to expand the microglial population in prion disease. It is also possible that during long term anti-IL34 treatment, CSF-1 will take over the function of IL-34 in maintaining and expanding the microglial population during neurodegeneration, given that it is also increased during disease. This possibility needs to be investigated in the future using more suitable pharmacological strategies than peripheral antibody administration.

Chronic inhibition of IL-34 did not majorly affect the abundance of blood monocytes or tissue-resident macrophage populations in liver and kidney, which were sensitive to CSF1R blockade, suggesting IL-34- independent mechanisms of survival, most likely through CSF-1. In line with this, a natural mutation in the *Csf-1* gene *(op/op)* caused a reduction in macrophages in most tissues of the body (12), and long-term treatment with a CSF1R-blocking antibody lead to a complete depletion of Kupffer cells in the liver and prevented the development of non-classical Ly6C^{lo} monocytes in the blood (37), which we likewise observed. It was previously shown, that genetic deficiency of IL-34 resulted in decreased numbers of microglia in most brain regions, while there was no effect on myeloid cells in blood and bone marrow, Kupffer cells in the liver, lung alveolar macrophages, and dendritic cells in the lung and spleen (20). Similarly, a specific impact of IL-34 blockade on Langerhans cell homeostasis, but not on liver, intestine and kidney macrophages after IL-34 antibody administration has been recently shown (32). We have also observed an effect on Langerhans cells, which were reduced after both CSF1R- and IL-34 antibody treatment, confirming a role of IL-34 in regulating their survival as well as the efficacy of anti-IL-34 antibodies. Overall, sensitivity of macrophage populations to IL-34 inhibition seems to be defined by the spatial expression pattern of IL-34, which rarely overlaps with CSF-1 expression. Thus, myeloid cells located in regions dominated by IL-34 expression can be targeted by inhibition of IL-34, which is restricted to fewer organs, potentially reducing unwanted side effects caused by therapeutic intervention targeting the CSF1R pathway. We propose that IL-34 inhibition could be a viable strategy to decrease proliferation of microglia in the context of neurodegenerative disease, with restricted impact on peripheral myeloid cells.

DATA AVAILABILITY STATEMENT

Requests to access the datasets should be directed to Diego Gomez-Nicola, d.gomez-nicola@soton.ac.uk.

ETHICS STATEMENT

The animal study was reviewed and approved by University of Southampton Ethics Review Group.

AUTHOR CONTRIBUTIONS

DG-N, VHP, DT, JB, HN, PA, EK, and CR conceived the study. DG-N supervised the project. JO prepared the figures and wrote the manuscript. ES, MM-E, EP, LB, IG-R, FB, AP, DF, and SF performed *in vivo* experiments and analyzed the data. All authors contributed to drafting the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.579000/full#supplementary-material

Supplementary Figure 1 | Gating strategy used for flow cytometric analysis of brain, blood, and bone marrow after CSF1R- and IL-34 antibody treatment.

Supplementary Figure 2 | Effect of CSF1R- and IL-34 antibody treatment on bone marrow. Macgreen mice were treated with anti-CSF1R or anti-IL-34 (both rat monoclonal IgG2A) by intraperitoneal injections of 250 μg antibody 3x a week for 3 weeks. **(A)** Flow cytometric analysis of $CSF1R^+$ cells in the bone marrow of anti-CSF1R- and anti-IL-34- treated mice did not result in significant alterations. Graphs indicate respective cell numbers per 5×10^4 $CD45^+$ cells. **(B)** Flow cytometry of $eGFP^+$ $Ly6C^{hi}$ and $eGFP^+$ $Ly6C^{lo}$ subpopulations of CSF1R-expressing cells in the bone marrow did not reveal any significant changes due to the treatment. Graphs indicate respective cell numbers per 5×10^4 $CD45^+$ cells. PBS $n = 8$, isotype $n = 8$, anti-CSF1R $n = 8$, anti-IL-34 $n = 7$, data shown represent mean ± SEM, two-way ANOVA followed by Tukey's multiple comparison test. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Supplementary Figure 3 | Distribution of CSF1R- and IL-34 antibodies in peripheral organs and brain. Macgreen mice were treated with anti-CSF1R or anti-IL-34 (both rat monoclonal IgG2A) by intraperitoneal injections of 250 μg antibody 3x a week for 3 weeks. Levels of rat IgG2a were measured in tissue lysates of brain, liver, kidney and spleen after the treatment by ELISA, showing no significant differences between anti-CSF1R and anti-IL-34 in individual organs. Brain: PBS $n = 8$, isotype $n = 8$, anti-CSF1R $n = 8$, anti-IL-34 $n = 7$, liver/kidney/spleen: PBS $n = 4$, isotype $n = 4$, anti-CSF1R $n = 3$, anti-IL-34 $n = 4$, data shown represent mean ± SEM, two-way ANOVA followed by Tukey's multiple comparison test. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

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REVIEW
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Microglia Diversity in Health and Multiple Sclerosis

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Multiple Sclerosis (MS) is a neurodegenerative disease characterized by multiple focal lesions, ongoing demyelination and, for most people, a lack of remyelination. MS lesions are enriched with monocyte-derived macrophages and brain-resident microglia that, together, are likely responsible for much of the immune-mediated neurotoxicity. However, microglia and macrophage also have documented neuroprotective and regenerative roles, suggesting a potential diversity in their functions. Linked with microglial functional diversity, they take on diverse phenotypes developmentally, regionally and across disease conditions. Advances in technologies such as single-cell RNA sequencing and mass cytometry of immune cells has led to dramatic developments in understanding the phenotypic changes of microglia and macrophages. This review highlights the origins of microglia, their heterogeneity throughout normal ageing and their contribution to pathology and repair, with a specific focus on autoimmunity and MS. As phenotype dictates function, the emerging heterogeneity of microglia and macrophage populations in MS offers new insights into the potential immune mechanisms that result in inflammation and regeneration.

Keywords: microglia, macrophages, single-cell analysis, single-cell RNA sequencing, multiple sclerosis, remyelination, ageing

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INTRODUCTION

Microglia are a specialized population of myeloid cells in the brain and spinal cord, and depending on the species and anatomical region, account for 0.5–16.6% of total central nervous system (CNS) cells (1, 2). Under homeostatic conditions they are the primary macrophage-like cell in the CNS. To maintain homeostasis microglia act as sentinels, continually surveying their environment by extending and retracting their motile processes, ready to respond to the first signs of pathogenic invasion or tissue damage (3). In the event of inflammation, microglia help orchestrate the immune response, balancing the risk of potential harm to delicate CNS tissue and supporting tissue repair and remodeling. The central role of microglia in the defense and maintenance of the brain and spinal cord implicates them in nearly all brain pathologies (4).

Microglia are derived from the embryonic yolk sac and take residence in the CNS early in development (3). As the brain and spinal cord mature, microglia respond to the changing environment and, help shape CNS tissue development; microglia contribute to the remodeling of postnatal neural circuits and play a role in synaptic pruning during postnatal development (5, 6). This bidirectional communication with the CNS during development helps establish a unique microglial identity. Once established, microglia density is sustained by balancing microglia proliferation and cell death, without a contribution from blood-derived cells (7).

In the last decade, advancements in technologies such as single-cell RNA sequencing and lineage tracing has shed light on the way microglia function under steady state conditions and during disease. Lineage tracing and genetic fate mapping allow microglia to be distinguished from other macrophage-like cells, which becomes crucial during pathological conditions as monocyte-derived cells enter the CNS parenchyma from the periphery and the two cell types become virtually indistinguishable from one another using classical markers (8). Techniques such as MARS-Seq and Drop-Seq, among many others, allow gene expression to be analyzed at the single-cell level. The ability to focus on microglia explicitly, combined with single-cell sequencing has allowed greater insight into cell trajectories, cell states, gene networks, and receptor-ligand interactions. This information supplements what is known about microglia across the lifespan, during development and during disease, including autoimmune demyelinating disorders such as multiple sclerosis (MS).

The pathological hallmark of MS is the formation of demyelinating lesions in the brain and spinal cord (9). These focal lesions are ubiquitously associated with the infiltration and activation of immune cells. Microglia are among the first responders and remain within lesions until the lesion resolves or becomes inactive. The lesion microenvironment changes over time and differs with anatomical location—i.e. white matter versus grey matter. The presence or absence of remyelination further complicates the lesion environment. Microglia are influenced by these changing lesion environments and are tasked with responding to the associated complex immune milieu. Understanding various microglia functions in MS lesions may help develop therapeutic interventions that tip the scale of the immune response towards repair and regeneration and away from tissue damage.

In this review, we discuss what is known about microglia origin and development; similarities and differences between human and murine microglia; and microglia heterogeneity throughout life, in the context of CNS autoimmunity and during remyelination and ageing. The heterogeneity of microglia during development, across the lifespan and in MS offers new insights into the potential immune mechanisms resulting in tissue inflammation or tissue regeneration.

MICROGLIA ESTABLISHMENT IN THE CNS

Microglia are CNS resident macrophages of the mononuclear phagocyte system (10). Under steady-state conditions, they are the primary resident myeloid population in the brain and spinal cord. Microglia first appear in early development (~E9.5 days post-conception) from a population of primitive macrophages that mature from mesodermal erythromyeloid progenitors in the embryonic yolk sac (11, 12). These primitive macrophages do not require the transcription factor Myb for their development, unlike monocyte-derived macrophages and those of the hematopoietic stem cell lineage (13). Initially, the primitive macrophages that give rise to microglia lack the classic leukocyte marker (Cd45) and express the receptor tyrosine kinase C-kit. They progressively lose C-kit expression while gaining expression of Cd45 as they mature (12, 14). These cells migrate through the developing vasculature to the brain rudiment, where they differentiate into microglia (13, 15). This migration starts around E9.5 in mice. Once inside the CNS, microglia undergo extensive local proliferation and spread out to populate the entire developing brain, ultimately acquiring their unique identity in tandem with neural tissue development (16).

Murine microglia isolated from various life stages reveal a progressive change in gene expression pattern that occurs in parallel with the developing brain as they influence and adapt to the changing CNS environment (17, 18). This reciprocal interaction between the developing brain and the maturing microglia population heavily influences the establishment of a unique microglia identity. Microglia identity is driven, in large part, by the activity of the critical lineage dependent transcription factors, Pu.1 and C/ebp (19). Mice lacking Pu.1 do not develop a microglia population (13). Other critical regulators of microglia identity include signal-dependent transcription factors such as Maf, Mef2c, Sall1and Irf8 (15, 20–22).

Local CNS factors maintain a healthy microglia population. Signalling through the colony-stimulating factor 1 receptor (Csf1r) is vital for microglial survival in mice, both developmentally and throughout the lifespan (23). Csf1 and Il-34 are the two known ligands for Csf1r that are both found in the CNS. Interestingly, microglia in white matter, grey matter and from distinct brain regions differ in their reliance on either Il-34 or Csf1 (24, 25). In the mature mouse brain, Tgf-β is another key regulator of microglia identity through the activity of Smad transcription factors (26, 27). During embryonic and early postnatal development, where there are high levels of microglia proliferation, Tgf-β is also a crucial contributor (28). Following a burst of postnatal proliferation, microglia self-renew slowly in a stochastic manner where the processes of proliferation and apoptosis are tightly coupled (7). While the exact rate of turnover is yet to be agreed upon, it is apparent that there are different rates of microglia turnover depending on brain location (7, 29) where human microglia divide on an average of 4.2 years, but some may not divide for over 20 years (30). Mouse microglia turnover approximately every 15 months (31).

The CNS contains other immune cells that may regulate microglia function. Outside the CNS parenchyma resides several distinct myeloid cell populations including perivascular, meningeal and choroid plexus macrophages. These populations are collectively known as border associated macrophages (BAM) (32, 33) or CNS-associated macrophages (CAM) (34).

Heterogeneity between and even within BAM populations has recently been uncovered and their roles in mediating immune cell entry and activation of T-cells investigated (32, 35). BAMs display heterogeneity with respect to the expression of antigen presentation genes appearing postnatally, suggesting that BAM diversity is primarily shaped after birth, in part under the influence of microbiome-derived stimuli (36). The interactions between BAM cells and parenchymal microglia remains to be studied. Other immune cell populations reside in the cerebrospinal fluid such as lymphocytes, dendritic cells, neutrophils and monocyte-derived cells (32). These and peripheral blood-associated immune cells infiltrate the parenchyma during injury and under disease conditions to affect microglia function (37). The extent to which these cells exert a remote influence on microglia activity remains to be fully understood.

MURINE MICROGLIA HETEROGENEITY THROUGHOUT LIFE

The advent of new technologies has allowed the exploration of cell biology at the single-cell resolution. Previous genomic strategies such as bulk RNA sequencing were focused on investigating global gene expression changes. These bulk strategies measured the average gene expression across a population of cells, which presented significant limitations in cases where cell types are heterogenous or divided into several populations with potentially different functions (38). To overcome this, Tang and colleagues developed single-cell sequencing technologies that used a combination of PCR amplification and microarray tools (39). With the expansion of new tools such as, MARS-Seq (38), Drop-Seq (40), Smart-Seq (41), Smart-Seq2 (42), Cel-seq (43), CEL-Seq2 (44) and SCRB-Seq (45)—that have been reviewed extensively by others (46, 47)—it is now possible to determine the transcriptome of cells or nuclei at an individual cell level. The study of microglia with single-cell resolution has allowed significant advances with recent developments in bioinformatics (48), such as defining cell trajectories (49), deciphering cell states, constructing gene regulatory networks (50) and inferring receptor-ligand interactions (51, 52).

One important discovery from single cell transcriptomic work is the presence of different microglial populations that vary phenotypically across development and lifespan. Embryonic and postnatal development is characterized by several unique microglia populations not present in adults (53, 54). For example, at E14.5 there is a population of metabolically active microglia enriched with lactate dehydrogenase (*Ldha*), an enzyme involved in glycolysis that produces lactate (53). This population is also enriched with migration inhibitory factor (*Mif*), which is often associated with microglia during inflammation (8). These observations suggest an overlap between microglia populations in development with those found during inflammation. During development, microglia prune synapses, clear dead cells and regulate cell numbers (55), which may account for this microglial inflammatory signature.

During the transition from embryonic development into the early postnatal period, there is some phenotypic overlap in microglia populations (53, 54). A population of proliferative microglia are enriched during embryonic development and at early stages postnatally (53). Proliferative microglia were enriched with different cell cycle-related genes and were found in equal magnitudes at E14.5 and P4/5, but not at P30 (53), which parallels other work on the proliferation of microglia (7). These proliferative microglia express genes related to the DNA damage response (*Ankle1*, *Lig1*), histone mRNA decay (*Eri1*) and epigenetic function such as histones and chromatin modifiers (54), suggesting that proliferation is largely limited to the embryonic and early postnatal time points when the microglia population is established.

During the first three postnatal weeks another microglia population arises in developing white matter axonal tracts. This early postnatal period coincides with active myelination of the corpus callosum and cerebellum in mice (56, 57). Three independent groups have defined this interesting population of microglia. Wlodarczyk and colleagues found a population of microglia expressing Cd11c that are a significant source of Insulin-like growth factor 1 (Igf1) (58), an important survival factor that promotes myelin development (59). When *Igf1* was conditionally removed from these Cd11c microglial cells, there was reduced myelin gene expression (58), which is consistent with the finding that microglia regulate myelin development (60). Similarly, using single-cell RNA sequencing, Hammond and colleagues identified a population of microglia enriched in the developing axonal tracts they referred to as axonal tract microglia (ATM) (53). These ATMs were characterized by the distinct expression of genes related to lysosomal activation (*Lamp1*, *Cd68*) and possessed an amoeboid morphology (53). Microglia prune myelin sheaths in development (61), which may account for the amoeboid morphology and lysosomal activation characterizing ATM. Li and colleagues independently identified an equivalent population that they termed proliferative-region-associated microglia (PAM) (54). These amoeboid PAMs preferentially phagocytosed fluorescently labelled beads relative to other microglia phenotypes (54). PAMs were found to engulf newly formed oligodendrocytes, which incur significant cell death upon the onset of CNS myelination (62). The emergence of the PAM phenotype coincides with myelination onset and, therefore, may play an essential role in clearing the overproduced oligodendrocytes (63). PAMs also upregulated genes associated with lipid metabolism, lipid transport and lysosomal acidification, presumably necessitated by the phagocytosis of lipid-rich oligodendrocytes (54). The CD11c (58), ATM (53) and PAM (54) all contained common distinguishing genes such as *Spp1, Igf1* and *Gpnmb*, suggesting these populations are the same. Although, a comparison of these populations is needed to confirm the extent of overlap (**Figure 1**).

Microglia diversity decreases after puberty, when microglia become more homogenous with fewer distinct phenotypes but with considerable variance in expression levels of homeostatic

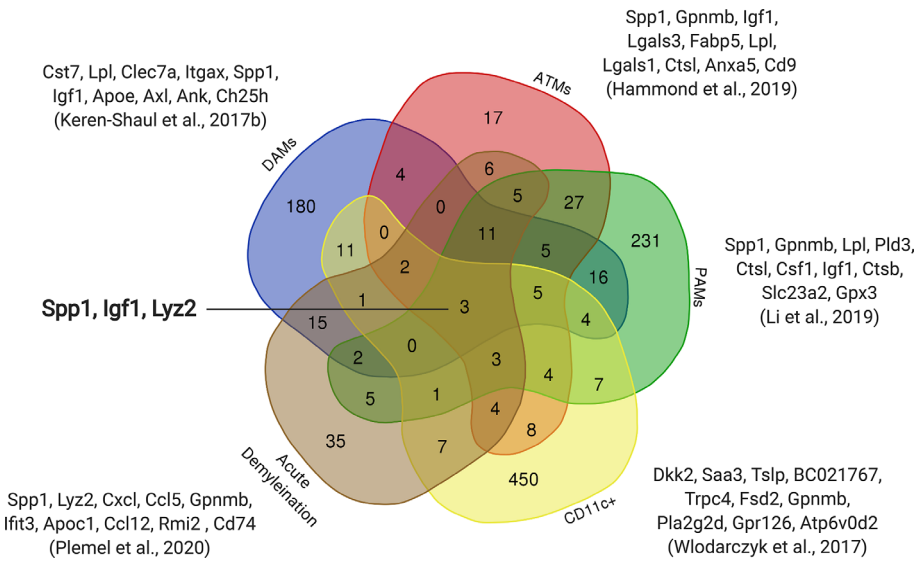

FIGURE 1 | Overlap of upregulated genes between early postnatal microglia and microglia in diseased models. Subsets of early postnatal microglia (ATMs, PAMs, Cd11c+) with similar transcriptomic profiles were observed in three independent studies (53, 54, 58). The transcriptomic profile of an Alzheimer's model (DAMs) (64) and an acute demyelination model (8) also show overlap with these postnatal microglia. The top ten upregulated genes from each dataset are shown with three genes that are common to all five datasets (*Spp1, Igf1, Lyz2*).

genes (**Figure 2**) (17, 53, 54). Adult homeostatic microglia are characterized by genes such as *Fcrls, Clqa, Selplg and Tmem119* (17, 27, 66, 67). Interestingly, the previously thought canonical microglia markers *(P2ry12, Cx3cr1, Tmem119)* are not found to be uniformly expressed across all homeostatic clusters and therefore may not be a robust way to detect microglia *in vivo* (53). The transition of microglia from the postnatal phenotype to the adult is dependent upon the transcription factor Mafb,

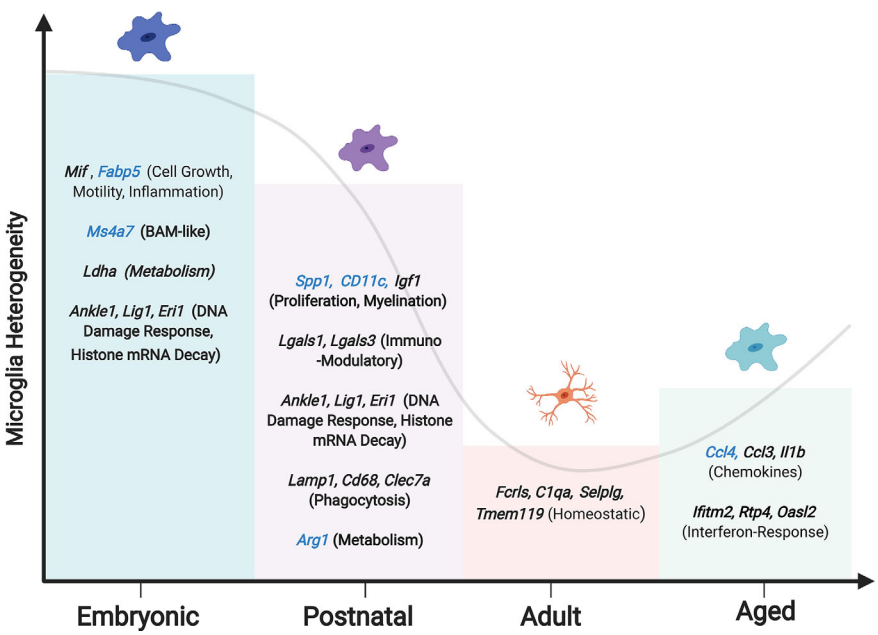

FIGURE 2 | Changing microglia heterogeneity throughout development. Peak microglial heterogeneity is seen during embryonic development, with a decrease in adulthood, and a subsequent increase in the aged brain (65). Enriched genes and phenotypes significant to each microglial developmental stage are shown, with genes that are unique to each stage in blue. Hammond et al. reported a subset of embryonic microglia uniquely expressing *Ms4a7*, suggesting a similarity to BAMs.

without which microglia upregulate antiviral genes and lose their homeostatic nature (17).

Regional differences in microglia phenotypes may reflect their functional requirements (68). For example, the cerebellum has a high neuronal turnover rate compared to the striatum and has been found to house a microglia subset that appears to specialize in debris clearance and apoptotic cell detection (**Figure 3**). This subtype is characterized by the presence of the genes more commonly associated with inflammation, such as *Axl, Apoe, Cd74*, and MHC-I genes. As the striatum consists of a neuronal population that is relatively stable throughout adulthood, it does not require a phagocytic microglia phenotype and is therefore accompanied by a homeostatic microglia phenotype lacking expression of activation genes (69). The deep brain structures also showcase a distinct variety of microglia. Microglia in the basal ganglia nuclei differ in their densities, morphologies and electrophysical properties (70). In the ventral tegmental area of the basal ganglia, genes related to metabolism are depressed and those required for growth factor release and phagocytosis are upregulated.

Age-related changes to microglia populations occur and suggest an overall heightened inflammatory response with regional variability (68). While whole-brain analysis demonstrates a significant overlap between microglia populations in young and aged mice, there is an expansion of two microglia populations enriched in the aged brain (53). These age-associated microglia populations are either enriched in the chemokine *Ccl4*, lipoprotein lipase (*Lpl*) or genes associated with interferon response such as *Ifitm4, Ifit3*, and *Irf7*. With age, microglia accumulate myelin fragments within lysosomal structures (71), which likely account for new age-related microglial populations. The dominance of inflammatory subpopulations may contribute to progressive neurodegeneration, which is often age-dependent (53, 54, 72).

SIMILARITIES BETWEEN HUMAN AND MURINE MICROGLIA

The similarities and differences between murine and human microglia have been explored in more detail elsewhere (27, 65). Here, we will briefly review recent work that has combined single-cell RNA sequencing with multiplexed mass cytometry and comprehensive histological analysis to explore species-specific microglia heterogeneity (73–75). The study of human microglia is challenging due to the relative scarcity of non-pathological human brain tissue. However, recent studies have taken advantage of microglia isolated from post-mortem brains of donors without diagnosed neurological disease and from tissue resected during the treatment of epilepsy, brain tumours and acute ischemic stroke that is isolated from outside the area of pathology and deemed histopathologically normal.

Human microglia have not been extensively studied at the embryonic level; however, studies by Zhong et al. and Kracht et al. corroborate mouse data, suggesting there is a higher level of heterogeneity in the gestational period, which culminates in microglia acquiring a more homeostatic phenotype (76, 77). Like mice, human microglia can be differentiated based on their developmental stage, suggesting there is a progressive developmental program for human microglia development. At early gestational weeks nine through eleven microglia are enriched in genes such as *ITGAX, CLEC7A, AXL*, and *PKM*, while the later gestational weeks, fifteen to seventeen, are enriched with more canonical microglia genes (*CX3CR1, TMEM119, P2RY12*). Functions have yet to be ascribed to these phenotypes, but initial steps have been taken to compare microglia clusters to functions based on gene ontology designations (77).

To compare and contrast microglia heterogeneity within and between species, Masuda and colleagues sequenced 3,826 microglia from healthy and injured (facial nerve axotomy and cuprizone) mouse brains in addition to 1,180 human cortical microglia and 422 CD45+ cells from MS brain tissue (78). While some of the homeostatic genes translated well between mouse and human (*Cst3, P2ry12, Tmem119, Emr1*), human microglia were found to be more diverse and had clusters with higher expression of chemokines (*CCL2, CCL4*) and distinct transcription factor profiles (*EGR2, EGR3*) (78–80). This study identified homeostatic human microglia clusters with distinct profiles, but also profiles that partially overlap with those of murine microglia. This same group further explored microglia heterogeneity across 18 different species using an extensive dataset that included 1,069 human microglia. They reported significant microglial heterogeneity in humans compared to all other mammals (75).

Using both single-cell RNA sequencing and mass cytometry, Sankowski and colleagues observed both age and spatial (white vs grey matter) heterogeneity in human microglia (81). Enriched in humans is a microglia population

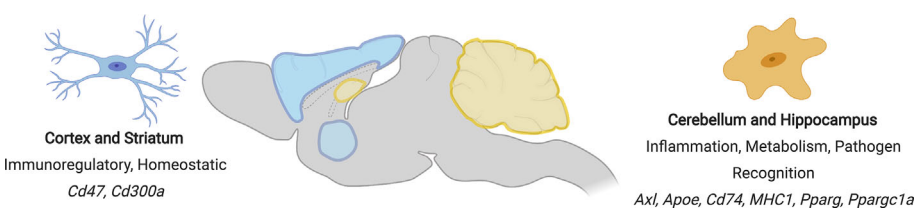

FIGURE 3 | Regional differences in microglial phenotypes in the mouse brain. Regions showing similar microglial phenotypes are similarly coloured (cortex and striatum = blue, cerebellum and hippocampus = yellow). Phenotypic characteristics and signature genes for each region are shown.

expressing the gene *SPP1* that encodes for a proinflammatory cytokine, osteopontin (78, 82, 83). In people under the age of 30, the proportion of microglia expressing *SPP1* is negligible. However, people over the age of 50 show a five to ten-fold increase in microglia expressing *SPP1*, suggesting microglia become more inflammatory as one ages. The human age-associated proinflammatory microglia are synonymous with inflammatory profiles identified in mice. In the same study, comparisons between white and grey matter were also made, highlighting the upregulation of the MHC-II antigen presentation complex related genes, *CD68* and *HLA-DR* in the white but not the grey matter (81).

Regional variability in microglia signatures was further explored using multiplexed mass cytometry of human microglia (84). Bottcher and colleagues analyzed microglia expression across five brain regions and found two prominent patterns (84): microglia located in the temporal and frontal lobe were defined by low levels of the mannose receptor CD206, whereas those in the thalamus, subventricular zone and cerebellum had no expression of CD206. These CD206 low microglia were distinct from what are presumably perivascular macrophages that expressed high levels of CD206. This study also found that microglia express similar genes in the fresh and post mortem isolates, albeit at slightly different levels, which validates the use of post mortem tissue in the study of human microglia signatures (84).

There are some common findings concerning microglia density in both mice and humans, with higher microglia density in the white matter than grey matter. Other similarities include relatively lower densities in the cerebellar cortex compared to regions of high density, such as in midbrain and brainstem structures (2, 75, 85). Despite these commonalities, overall microglial density varies markedly between the two species. Reported differences include a higher microglial density in the frontal cortex of mice compared to humans and more microglia in the human cerebellum and hippocampus compared to mice (75). Despite these differences in density, the morphological features of microglia remain relatively similar, including branch points, terminal points and dendrite length. The functional importance of these species' differences and the effect that these differences might have on our understanding of microglia during neurological diseases such as MS remains to be fully elucidated.

MICROGLIA/MACROPHAGE HETEROGENEITY IN THE CONTEXT OF CNS AUTOIMMUNITY

In MS, microglia and macrophage likely serve diverse roles and acquire distinct phenotypes given the variable nature of the disease. MS is characterized by demyelinating lesions along with progressive degeneration of white and grey matter (86, 87). In the active stages of MS—with the presence of new lesions—there is a dissemination of lesions in anatomical space and over time. At any given moment, a person with MS is likely to have old and new lesions in both the grey and white matter regions; these differences affect the pattern of microglial gene expression (88). The potential effects of lesion evolution on microglial/macrophage phenotypes are compounded by the presence of myelin regeneration, or remyelination. Microglia found in demyelinating and remyelinating conditions possess different phenotypes (78), with demyelination-associated microglia resembling patterns found associated with neurodegenerative disease (64). In parallel with lesion formation, MS is characterized by ongoing neurodegeneration that is often measured by advancing brain atrophy (89). Overall, the microglia and macrophage phenotypic heterogeneity and their diverse responses are likely related to temporal differences in lesion progression coupled with potential ongoing remyelination or neurodegeneration and interactions with other cell types. Regional variability in microglia phenotypes in the non-diseased state is likely to add complexity in the immune response during MS with disease characteristics convolving onto regional disease heterogeneity (**Figure 4**) (88). Animal models are designed to replicate different features of the disease to understand various aspects of MS. In this section, we focus on microglia and macrophage's role during the autoimmune attack in the CNS. Much of what we know about the mechanism of lesion formation and evolution comes from the experimental autoimmune encephalomyelitis (EAE) model.

TOXICITY OF MICROGLIA AND MONOCYTES DURING EAE

In EAE, various myelin antigens are given to a mouse in conjunction with an adjuvant to stimulate a myelin mediated autoimmune response [reviewed by (90, 91)]. Although there is

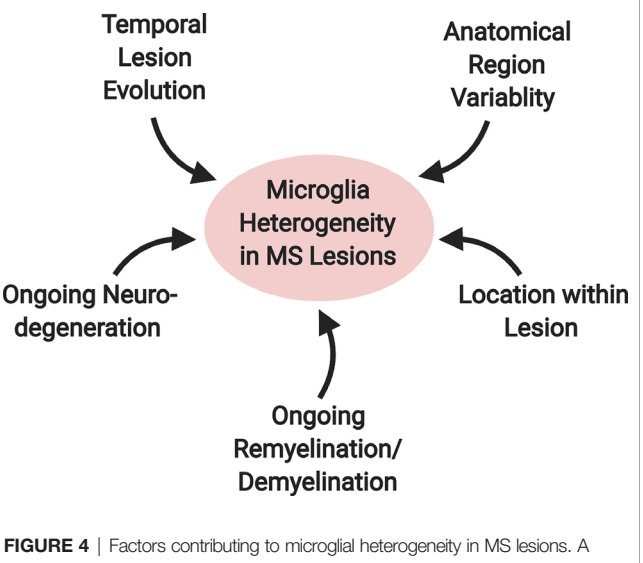

FIGURE 4 | Factors contributing to microglial heterogeneity in MS lesions. A variety of factors likely contribute to the diversity of the microglial phenotype in MS. These include: 1) temporal lesion evolution, 2) ongoing neurodegeneration and 3) remyelination/demyelination in the surrounding environment, 4) location within the lesion and 5) anatomical location within the brain.

variability between models, therapeutics that prevent T-cell activation or trafficking prevent EAE (92, 93). Despite T-cells' critical role in initiating autoimmune injury, T-cells collaborate with microglia and macrophages to induce toxicity (94). For example, Heppner and colleagues used transgenic mice expressing the suicide gene thymidine kinase under the expression of the *Itgam* (*cd11b*) promoter to kill myeloid cells and found that ablation in these mice considerably repressed EAE (95). Similarly, the removal of Tak1—an NF-κB cell signalling mediator—from microglia and BAM almost completely prevented autoimmune injury (96). Selective removal of Tak1 prevented demyelination but also dramatically suppressed T-cell infiltration into the CNS suggesting that microglia or BAM regulate lymphocyte trafficking into the CNS during EAE. Monocyte-derived macrophages are also required for the autoimmune injury during EAE. Monocytes are elevated in the blood before an increase in disability and monocytes' entry into the CNS triggers EAE progression (97, 98). Preventing monocyte entry by removing the chemokine receptor Ccr2 reduces clinical disability and toxicity in the CNS during EAE, suggesting that monocyte-derived macrophages are toxic (97, 99). Taken together, the combined efforts of microglia, BAM and monocyte-derived macrophage are required in the pathogenesis of EAE and likely contribute to lesion formation and evolution in MS.

It is still unclear whether these cells induce direct toxicity, or whether they act through indirect mechanisms. For example, while both microglia and macrophage produce reactive oxygen species (ROS) during EAE, a greater proportion of monocyte-derived macrophages express ROS producing enzymes than microglia (100). The production of ROS by phagocytes during EAE produces injury to myelin and axons alike, and can be diminished with ROS and reactive nitrogen species (RNS) scavengers (101, 102). Other direct mechanisms of toxicity by microglia and macrophage include the release of glutamate (103–105), or the expulsion of numerous potentially toxic cytokines (100, 106, 107). The toxic properties of microglia or macrophage may also be indirect. Microglia prevent the migration of infiltrating macrophages into spared tissue (8), and may also serve important "gate-keeping" functions for other leukocytes that are toxic during EAE. The roles of microglia are likely to evolve throughout the disease, as demonstrated by the finding that microglia ablation with a Csf1 inhibitor during EAE progression accelerates clinical disability (108).

MICROGLIA HETEROGENEITY DURING EAE

Despite the hundreds of receptor systems expressed by microglia (109), their activation and response to damage does have similarities across disease conditions. For example, microglia in an environment of amyloid induced neurodegeneration form a disease-associated microglia (DAM) (64), characterized by the downregulation of canonical microglial genes (*P2ry12/13*, *Cx3cr1*, *Tmem119*, *Cst3*) and upregulation of genes mapped to lipid metabolism pathways and phagocytosis (*Apoe*, *Lpl*, *Cst7*, *Ctsd*, *Tyrobp*, and *Trem2*). Certain genes, such as *Hexb* are stably expressed in homoeostatic microglia, DAM, and other neurological conditions (64, 110). Elements of this DAM signature were later observed in microglia activated by diverse conditions such as following white matter injury (8, 53), EAE (35), MS (78, 82), amyloid lateral sclerosis (ALS) (64, 111), ageing (53, 111), facial nerve injury (112) and cancer (81). Krasemann and colleagues analyzed gene expression patterns from microglia isolated during Alzheimer's disease, EAE and ALS mice models and identified a common microglia response (111). The microglia response to neurodegeneration required lipid receptor and trafficking elements *Apoe* and *Trem2* under diverse disease conditions, suggesting that some aspects of microglia activation in murine disease models are conserved. Critical aspects of this microglia signature were stimulated by the injection of apoptotic neurons that were later engulfed by microglia. The typical microglia response to diverse disease conditions may be a consequence of clearing debris, dead cells or other neurodegenerative molecular patterns (113).

Despite a common microglia response to disease, there is also a diversity in the microglia response during EAE and MS (35, 78). Jordao and colleagues identified four different clusters of disease-associated microglia in mice induced with EAE (35). EAE microglia were enriched with a phenotype characterized by markers of inflammation and proliferation *Ly86, Ccl1, Cxcl10, Mki67, Ccl4*, and *Ccl5*. The *Ccl5* and *Cxcl10* provide more of an understanding of this phenotype as these chemokines aid in leukocyte recruitment, which could be a potential future avenue to explore (35). They also identified a heterogenous response by other CNS resident macrophages such as those from the leptomeninges, the perivascular space and choroid plexus (35), suggesting a CNS-wide transcriptional change during autoimmune-mediated CNS injury. Ajami and colleagues also identified a population of CNS associated macrophages enriched in expression of diverse cytokines that were not found in healthy mice and peaked during symptomatic EAE (114).

Diverse populations of microglia were also found in the MS brain (78). Three subsets of MS-specific microglia were identified. Subsets were enriched for *SPP1* or *CD74*, which also defined microglia from mice given the demyelinating agent cuprizone and isolated under either demyelinating or remyelinating conditions, respectively (78). The transcriptional signature of microglia may one day be used to determine whether a lesion is demyelinating or remyelinating. The MS lesion exhibits marked diversity: Park and colleagues used imaging mass cytometry to examine the heterogeneity of CNS-associated macrophages and found that their diversity could be stratified based on their relative location within the MS lesion with enriched lysosomal LAMP1 or receptor tyrosine kinase MERTK expression on myeloid cells located at the lesion rim (115, 116). Taken together, microglia initiate certain conserved activation patterns in diseased conditions but also, microglia exhibit several unique phenotypes likely reflecting their local environment. Understanding the function and ubiquity of disease-specific microglia phenotypes will provide a greater understanding of neurological diseases.

MONOCYTE HETEROGENEITY DURING EAE

Monocyte diversity in the CNS similarly increases during autoimmunity. Ajami and colleagues identified five subsets of monocytes in the CNS that changed their expression profile throughout EAE (114). Peak EAE is defined by the simultaneous expression of three or four different cytokines in a given cell, not found in homeostatic subsets. By comparing the surface markers of blood-derived myeloid cells to the CNS-resident macrophage, Ajami and colleagues identified a new cell surface marker, Cd49e—or α5 integrin—that is upregulated by infiltrating monocytes (114). Treatment with antibodies that blocked Cd49e delay the onset and reduced the severity of EAE. Using single-cell RNA sequencing, Giladi also examined monocyte and monocyte-derived macrophage diversity during EAE finding eight distinct populations (117). Using antibodies against Ccr2 to ablate monocytes and reduce EAE severity, Giladi and colleagues identified two distinct monocyte populations that were selectively lost, and presumably are pathogenic given their association with disease conditions (117). Surprisingly, monocyte depletion resulted in minor changes to other immune cells suggesting monocytes may be pathogenic due to direct cytotoxicity. Given the toxic role of monocyte-derived macrophages, understanding monocyte diversity—with particular focus on pathogenic populations and how they traffic into the CNS—will lead to new macrophage focused therapies.

MICROGLIA/MACROPHAGE POPULATIONS DURING REMYELINATION

Myelin injury is a crucial attribute of demyelinating diseases such as MS, but so is the regeneration of myelin, or remyelination. For people with MS, remyelination occurs, but it is highly variable and prone to failure (118–122). Remyelination can restore lost behaviour due to myelin injury (123) and protects axons from degeneration (124)—which causes irreversible harm that is thought to contribute to ongoing progression. Indeed, promoting remyelination spares axons and improves functional recovery following EAE (125). For these reasons, finding therapeutic agents that promote remyelination is an exciting new avenue to treat MS. Several clinical trials are ongoing but no therapies have been approved as of yet (122, 126). Remyelination requires a favourable immune response from macrophage/microglia to clear inhibitory myelin debris and secrete growth factors and cytokines, such as Igf1 and activin-A, that regulate remyelination and the extracellular matrix (127–129). Despite the many benefits of the immune response to remyelination, there are only a few strategies that focus on improving the immune response as a means to boost remyelination (130–133). The paucity of immune-boosting targets in MS likely reflects the challenges of promoting immune activities because there are numerous immune-mediated mechanisms of neurotoxicity that could potentially be triggered.

Pioneering work by Miron and colleagues demonstrate that microglia/macrophages take on a proinflammatory signature early after demyelination that promotes OPC proliferation (134). These proinflammatory macrophage/microglia secrete cytokines such as Il1β and Tnf, which stimulate OPC survival and proliferation (135, 136). The proinflammatory microglia/macrophage then transition to an immunoregulatory phenotype (134). Ablation of these immunoregulatory immune cells contributes to remyelination failure suggesting that the transition from the proinflammatory state to an immunoregulatory one is an important step during remyelination. Unknown from this work is whether microglia or macrophage express these proinflammatory or immunoregulatory factors. Research from our group shows that the classic proinflammatory (iNos) and immunoregulatory markers (Arg-1) used by Miron and colleagues are not expressed by microglia following LPC mediated demyelination of the spinal cord (8). The proinflammatory and immunoregulatory phenotypes described by Miron and colleagues may therefore be attributed to blood-derived macrophages. Recently, Lloyd and colleagues investigated how microglia/macrophage transition from a proinflammatory to an immunoregulatory phenotype (127). Surprisingly, this transition required necroptosis, a form of programmed necrosis. Inhibiting necroptosis stalled remyelination and maintained high levels of proinflammatory microglia/macrophages, suggesting that necroptosis regulates the shift away from the proinflammatory phenotype.

While microglia and macrophages can take on proinflammatory and immunoregulatory phenotypes, new deep phenotyping of immune cells suggests that there are more diverse immune states after demyelination. We identified three distinct microglia phenotypes by isolating microglia following LPC-mediated demyelination of the spinal cord and conducting single-cell RNA sequencing (8). Microglia were isolated five days after LPC demyelination–a time point before remyelination characterized by OPC recruitment (137–139). We found that most activated microglia were enriched for *Spp1*, or osteopontin, *Apoe*, and *Cd74* (8). These genes are commonly expressed in microglia within the diseased, neurodegenerative CNS (64) and may, therefore, reflect microglia that are responding to damage or neurodegenerative molecular patterns (113). We also found a population of microglia enriched in interferon associated genes such as *Ifit3*, *Irf7*and *Ifitm3* as well as a third population likely reflecting proliferative microglia (8). At seven days after LPC demyelination of the corpus callosum, Hammond and colleagues similarly used single-cell RNA sequencing and identified similar populations of microglia, suggesting the microglial response may be consistent between these regions (53). Yet to date, none of the deep sequencing studies to date have investigated how microglia or macrophages change throughout the continuum of remyelination. This work could identify yet more states of immune cell activity.

The tools for differentiating microglia and macrophages are relatively novel and understanding the regenerative and neurotoxic aspects of these cell types is an area of research still in its infancy. Remyelinating models are valuable tools to understand the beneficial aspects of the immune response. After all, remyelination is perhaps the clearest example of regeneration in the CNS and likely resembles regenerative

processes in other tissues that also depend on a tightly regulated inflammatory response. Presumably, the immune cell phenotype will inform its function; therefore, identifying remyelination associated microglia and macrophage phenotypes are vital. Identifying immune cell phenotypes may also provide new biomarkers for remyelination. In MS, microglia/macrophages make up the majority of immune cells within the lesion (140) and MS lesions classification often relies on the presence and location of activated microglia/macrophage (141). However, activated microglia/macrophages are enriched during ongoing CNS injury (142) and can present during remyelination (143, 144). The accumulation of microglia/macrophage is, therefore, not a sensitive predictor of injury or regeneration. Given that microglia and macrophage are highly plastic and take on a unique cell state in response to diverse disease conditions, these cell states may indicate the stage or relative toxicity of the immune response. Indeed, the phenotype of microglia during active demyelination is distinct from microglia during remyelination (78).

AGE-ASSOCIATED REMYELINATION DECLINE INVOLVES IMPAIRED MICROGLIA/MACROPHAGE RESPONSE

It has been known for almost three decades that the efficiency of remyelination declines with ageing (145, 146). Given that remyelination protects axons from degeneration (124), preventing remyelination decline due to ageing may slow MS neurodegeneration. Mechanisms underlying this age-related impairment have been attributed to both CNS-intrinsic and extrinsic factors (147). For example, extrinsic factors such as the inadequate clearance of myelin debris in aged mice are restored by a more youthful peripheral immune response (148). Interestingly, the ageing demyelinated lesion increases in stiffness, potentially due to the extracellular matrix remodelling functions of aged microglia/macrophage (149), which impairs remyelination (150).

As activated microglia and infiltrating macrophages play an essential role in remodelling the lesion microenvironment, the changes these cell types undergo with ageing have a direct impact on the age-related impairment in remyelination efficiency. One of the first studies to document this link observed a delay in the expression of several essential growth factors following demyelination in ageing animals (151). This alteration in Pdgfa, Tgf-β, and Igf1 was associated with a delay in recruiting macrophages and microglia to lesions in ageing rats (152). In addition to this dysregulation in growth factor kinetics, lesions from ageing rodents displayed an accumulation of inhibitory myelin debris, suggesting that macrophages' and microglia's phagocytic capacity becomes impaired with ageing (148). Several studies have now highlighted a deficiency in the ability of ageing microglia and macrophages to phagocytose myelin debris (130, 133, 153). These alterations have been attributed to a disruption in retinoid X receptor signalling and a decrease in the expression of the scavenger receptor Cd36 (130, 133). In addition to deficiencies in the initial engulfment of myelin debris, another group identified disruptions in the lysosomal processing and subsequent cholesterol efflux of ingested myelin (71, 131). Accumulation of lysosomal inclusions and cholesterol crystals in ageing microglia resulted in inflammasome signalling and proinflammatory cytokine expression, resulting in a lesion microenvironment not conducive to efficient regeneration.

Due to difficulties distinguishing microglia from monocyte-derived macrophages within the lesion, no studies to date have been able to assign intralesional functional differences between these two cell populations with ageing. The advent of phenotypic markers and genetic fate-mapping strategies to distinguish these two populations opens up a promising new avenue of inquiry (110, 154, 155). Circumstantially, it has been documented that the ageing process manifests differently in microglia compared to monocyte-derived macrophages. As microglia are self-renewing cells within a CNS microenvironment that accumulate myelin fragments and protein aggregates with advancing age, they assume a senescent phenotype that is "primed" (71, 156). Single-cell sequencing of microglia from the ageing brain shows the expansion of two different clusters that upregulate several inflammatory signals such as *Ccl4, Il1b*, as well as several interferon-response genes (53).

In contrast, ageing monocyte-derived macrophages display an impairment in producing a functional proinflammatory cytokine response when stimulated with potent activating agents such as LPS (157). As the half-life of circulating monocytes in humans is approximately 71 h, it is postulated that the age-related changes in monocyte-derived macrophages manifest at earlier stages in monocyte development, such as at the level of the hematopoietic stem cell (158, 159). In addition to differences in the manifestations of ageing between microglia and monocyte-derived macrophages, it is now appreciated that microglia from diverse regions within the CNS also age differently (68). Future studies using single-cell sequencing and genetic fate-mapping to dissect microglial and macrophage transcriptional and functional heterogeneity within lesions and in the context of ageing will be essential to establish better how best to target these cells therapeutically and promote myelin regeneration.

CONCLUSION

We are at the dawn of a new era in recognizing microglia heterogeneity. Research is accelerating to identify microglial phenotypes throughout development and disease. Work must continue to expand upon our understanding of the gene and protein expression of microglia during development, throughout life, at different stages of disease and in different spatial locations relative to damage as this research will advance our knowledge of microglial functions and the interactions between microglia and other cell types.

Defining microglia will provide new cellular and phenotypic markers that can be used to detect and manipulate microglia phenotypes in MS and other neurological conditions. As the primary innate immune cells of the brain and spinal cord, microglia are uniquely positioned to both exacerbate the injury and be neuroprotective or even reparative. Still, with only recently available tools to target microglia directly, much work remains to define microglia function in different conditions. In the field of MS, we must still differentiate the contributions of microglia, infiltrating macrophages and BAM during remyelination, progression and throughout autoimmune injury. The next frontier will be to resolve the functions of these different microglia phenotypes.

Important questions remain: are there neuroprotective or neurotoxic microglial phenotypes? If so, what factors promote these phenotypes? Can they be targeted therapeutically? The availability of serum-free cell culture models for murine (160) and human cells (161–164) will support the functional analyses of distinct microglial phenotypes. Newer single-cell sequencing modalities such as IN-seq (165) or CITE-seq (166) allow protein markers to be overlaid onto single-cell sequencing defined immune cell phenotypes, permitting comparisons of cellular signalling or state to immune phenotypes. Strategies such as Tox-seq are also available to differentiate one function—ROS production—and overlay this function onto immune cell clusters. Bioinformatic tools such as NicheNet (51) and CellPhoneDB (52) provide a way to identify new receptor-ligand pairs from single-cell RNA sequencing data, which will serve as the starting point to dissect intercellular communications between CNS macrophages and their surrounding cellular niche that can then be studied *in vitro* and *in vivo*. With these and other available tools, it will be possible to dissect functionally distinct microglial and macrophage phenotypes so that they can be manipulated in MS and other neurological conditions. This ability offers the potential to harness the immune system's capabilities and bias the CNS lesion environment towards protection and repair rather than damage.

AUTHOR CONTRIBUTIONS

SZ, KR, NM, BK, LH, and JP drafted and reviewed the manuscript. MB and SZ constructed the figures. JP supervised the drafting of this manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Immovable Object Meets Unstoppable Force? Dialogue Between Resident and Peripheral Myeloid Cells in the Inflamed Brain

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Inflammation of the brain parenchyma is characteristic of neurodegenerative, autoimmune, and neuroinflammatory diseases. During this process, microglia, which populate the embryonic brain and become a permanent sentinel myeloid population, are inexorably joined by peripherally derived monocytes, recruited by the central nervous system. These cells can quickly adopt a morphology and immunophenotype similar to microglia. Both microglia and monocytes have been implicated in inducing, enhancing, and/or maintaining immune-mediated pathology and thus disease progression in a number of neuropathologies. For many years, experimental and analytical systems have failed to differentiate resident microglia from peripherally derived myeloid cells accurately. This has impeded our understanding of their precise functions in, and contributions to, these diseases, and hampered the development of novel treatments that could target specific cell subsets. Over the past decade, microglia have been investigated more intensively in the context of neuroimmunological research, fostering the development of more precise experimental systems. In light of our rapidly growing understanding of these cells, we discuss the differential origins of microglia and peripherally derived myeloid cells in the inflamed brain, with an analysis of the problems resolving these cell types phenotypically and morphologically, and highlight recent developments enabling more precise identification.

Keywords: microglia, neuroinflammation, central nervous system infiltration, neuropathology, central nervous system infection, monocyte-macrophage

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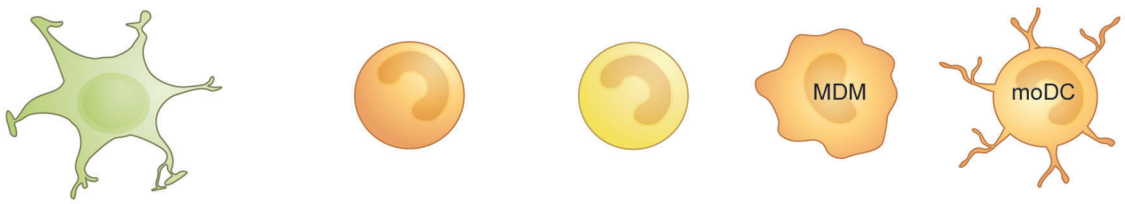
INTRODUCTION

Like other organs of the body, it is now well established that the central nervous system (CNS) has its own unique immune system that constantly maintains homeostasis and is rapidly engaged during inflammatory insult. Arguably, microglia are the key regulators of the immune response in the healthy brain. However, under certain conditions, such as those underlying neurodegenerative disease, autoimmunity, infectious encephalitis, and ischemia, infiltration of bone marrow (BM)-derived

monocytes act in concert with local microglia in the brain parenchyma to initiate, enhance, or dampen immune activity. Resident and infiltrating myeloid cells in the inflamed brain may be developmentally distinct, but often adopt similar morphologies and phenotypes, complicating accurate identification. More nuanced tools have improved resolution, and through these we can better define populations in the brain, allowing further elucidation of the role of resident and peripherally infiltrating myeloid cells in the inflamed brain. Given the fast-developing field, and the evident importance of both microglia and BM-derived monocytes to disease processes in a variety of CNS pathologies, we review the current understanding of the origins and functions of these cell types in homeostasis and highlight new experimental tools, molecules, and drugs which may overcome issues of differentiating between these populations during neuroinflammation.

MICROGLIA ORIGINS AND RENEWAL

Historically, microglia were first believed to be of neuroepithelial origin (1, 2), along with neurons and neuroglia. Subsequently, they were thought to be of monocytic origin (3), derived from hemopoietic stem cells (HSCs) in the fetal liver or BM. In 1999, Alloit et al. proposed the yolk sac (YS) origin of microglia (4). A decade later, this was confirmed using a fate-mapping model to trace YS progenitors, replacing the view of a monocytic origin for microglia (5). Microglia are now known to arise from uncommitted KIT$^+$ erythromyeloid precursors (EMP) (6) (**Figure 1**), which seed the brain from the YS at embryonic day 9.5 (E9.5) in the mouse (5), well before other glial cells and before the formation of the blood-brain barrier (BBB) (6, 7). However, other evidence suggests that microglia are not exclusively YS-derived, and that a small population arise from Hoxb8$^+$ progenitors in the E12.5 fetal liver (8) or from fetal HSC-derived monocytes (9). Subsequent to the formation of the brain, microglia are renewed *in-situ* throughout life, independently of BM-derived HSCs (10–12). In the steady state, microglia have region-specific renewal rates (13) with their density maintained *via* the tight coupling of apoptosis and proliferation (14). In the mouse brain, half the microglial population persists throughout the entire lifespan of the animal and thus remains a relic of the embryonic brain (11). In young and adult mice, the median life span of microglia is 22 and 29



	Microglia	Ly6C^{hi} monocyte	Ly6C^{lo} monocyte	Monocyte-derived cells
Progenitor:	Uncommitted KIT$^+$ erythromyeloid precursor	MDP, GMP, and cMoP	Ly6C^{hi} monocytes	Ly6C^{hi} monocytes
Location:	CNS parenchyma	Bone marrow and spleen	Circulation	Inflamed tissues
Phenotype:	CD11b$^+$, CD45^{lo}, CX_3CR1^+, P2RY12$^+$, F4/80$^+$, FCRLS$^+$, Siglec-H$^+$,TMEM119$^+$, SALL1$^+$, CD64$^+$, CD68$^+$, MERTK$^+$, CD115$^+$, SPARC$^+$	CD11b$^+$, CD45^{hi}, Ly6C^{hi}, CX_3CR1^{int}, CCR2$^+$, CD62L$^+$, CD43^{lo}, F4/80$^+$, MHC-II^a	CD11b$^+$, CD45^{hi}, Ly6C^{lo}, CX_3CR1^{hi}, CCR2^{lo}, CD62L$^-$, CD43$^+$, MHC-II^a	CD11b$^+$, CD45^{hi}, MHC-II$^{+/-}$, CD11c$^{+/-}$, Ly6C$^+$, CD64$^+$, CD115$^+$, BST2^b, CD172a$^+$, CD206$^+$, CD14$^+$, CD16$^+$, MERTK$^{+/-}$, FCεRI$^{+/-}$
Human equivalent:	CD11b$^+$, CD45^{lo}, CX_3CR1^{hi}, P2RY12^{hi}, CD91^{hi}, CCR5^{hi}, TGF-β1^{hi}, TMEM119^{hi}, TREM2^{hi} CD64^{hi},CD32^{hi}, CD172a^{hi},HLA-DR^{hi}, EMR1^{hi} CD11c^{hi}, CD115^{hi}	CD14$^+$, CD16$^-$ classical monocytes	CD14^{lo}, CD16$^+$ non-classical monocytes	CD11b$^+$, CD11c$^+$, HLA-DR$^+$, CD64$^+$, CD115$^+$, BDCA3$^+$, CX_3CR1^+, CD1c$^{+/-}$, CD172a$^+$,CD206$^+$, CD14$^+$, CD16$^{+/-}$, FCεRI$^{+/-}$, CD1a$^{+/-}$, Flt3$^{+/-}$, Zbtb46$^{+/-}$

$^{+/-}$ Heterogenous expression or tissue-dependent expression
^{a} Inducible
^{b} Activated

FIGURE 1 | Origins and phenotypes of resident microglia and monocyte-derived cells in the periphery and inflamed brain.

months, respectively (11). In humans, microglia can survive for more than twenty years, although unlike mice, their entire population is renewed at a median rate of 28% per year (10). The correlation of high differential renewal rates with microglial function remain to be revealed.

The microglial phenotype is derived from the successive development of uncommitted KIT$^+$ EMP into the macrophage ancestor population A1 (CD45$^+$, CX3CR1$^-$, F4/80$^-$) and then into the A2 progenitor population (CD45$^+$, CD115$^+$, CX3CR1$^+$, F4/80^{hi}), which migrates to and populates the embryonic brain (6). The development of microglia from precursor cells into intermediate progenitors is a finely tuned process orchestrated by external and internal stimuli. PU.1, RUNX1, and IRF8 are indispensable transcription factors in the programming of EMP into microglia during embryonic development (5, 6, 15, 16). CD115 (also known as colony-stimulating factor-1 receptor, CSF-1R, or macrophage colony-stimulating factor receptor, M-CSFR) ligands, CSF-1, and interleukin (IL)-34, are important for the maintenance of microglia in the adult brain, with IL-34 being highly expressed by neurons in a region-specific manner in the adult mouse brain (17, 18). CD115 signaling is more critical during development, playing an important role in the differentiation of EMP into microglia, but is also required for replenishment of adult microglia and maintenance (18–20). CD115-deficient mice have reduced microglial numbers, and treatment with CD115 inhibitors at high doses results in significant microglial depletion (19, 21).

Remarkably, microglia rapidly renew their entire population after chemical or genetic (conditional) depletion. Depending on the depletion method, the presence of non-physiological perturbations, and/or the experimental model, studies have suggested this occurs through niche repopulation by infiltrating monocytes, proliferation of a microglial progenitor or proliferation of surviving microglia. In the absence of BBB breakdown or lethal irradiation and BM transplant, it is believed that there is little or no contribution of HSC-derived monocytes to the microglial pool (12, 22) and that surviving microglia repopulate *via* self -renewal (23–25). Experimental methods used to deplete myeloid cells in the CNS and periphery are presented in **Table 1**. In irradiated BM-reconstituted CD11b-herpes simplex virus thymidine kinase (HSV-TK) mice injected intracerebroventricularly (i.c.v.) with ganciclovir to deplete microglia, engrafted "microglia" were of peripheral origin (45) (**Table 1**). On the other hand, following treatment with PLX3397, a small-molecule CD115 inhibitor, replacement microglia arose from a resident microglial progenitor population expressing nestin, a neural stem cell marker that can also be expressed on macrophages (19) (**Table 1**). By contrast, microglial depletion in either Cx3cr1CreER:iDTR mice, in which long-lived CX3CR1$^+$ cells (microglia) are depleted after tamoxifen and diphtheria toxin (DTx) administration (**Table 1**) (25) or with the CD115 inhibitor PLX5622 (24, 46), showed little contribution of nestin$^+$ progenitors or peripheral myeloid cells to the regenerating microglial pool, supporting the innate capacity for microglial self-renewal (**Table 1**). The specific attributes required for microglial survival (and thus incomplete depletion) during these depletion procedures are unclear, but there is an implied refractoriness in the pathways involved in surviving microglia reminiscent of a developmental stage difference or "stemness," with survivors clearly retaining the ability to proliferate for population renewal.

The concept that microglia are capable of self-renewal without input from peripheral myeloid cells, both in homeostasis and disease, was established in "microfetti" mice (*Cx3cr1*^{CreER} mice crossed with R26RConfetti reporter mice) and in a model of parabiosis. In microfetti mice, replenished microglia are tagged with one of four reporter proteins of the confetti labelling system, giving information on the distribution, expansion, and clonality of repopulating microglia. After unilateral facial nerve axotomy, microglia underwent rapid self-renewal with no contribution from progenitors or external myeloid populations (13). In the parabiosis model, a transgenic mouse expressing green fluorescent protein (GFP) in hemopoietic mononuclear cells and a wild type (WT) mouse were surgically attached for several weeks to achieve 50% blood chimerism (12). When the WT mice were subjected to facial nerve axotomy or amyotrophic lateral sclerosis (ALS), the CNS of the WT mice had no GFP$^+$ cells (partner-derived cells), demonstrating *in-situ* microglial repopulation.

While the capacity of microglia to self-renew without contribution from the periphery has emerged as the dogma, these ideas were established using parabiotic mice and mild inflammatory insults. During severe inflammatory insult and/or perturbation of the BBB, it was speculated that microglia could be derived from circulating peripheral monocytes (30). As the circulating myeloid compartment serves as a reservoir of immune cells that can rapidly be recruited to any tissue as needed, whether to contain virus or assist in tissue repair after traumatic injury, this may be an additional pragmatic solution to replenishing microglia, either in the short or long term, notwithstanding a likely differential genetic signature (47). It is unclear if such BM precursors are sufficiently stem-like to become "real" microglia once in the CNS, and if so, whether they could become a completely self-renewing immigrant population that can maintain a density and network configuration similar to native microglia. Whether such engrafted "microglia" would function similarly to YS-derived microglia in both homeostasis and pathology over time, is of considerable interest and still unresolved.

A DAY IN THE LIFE OF MICROGLIA: FUNCTIONS IN THE EMBRYONIC AND ADULT CNS

The importance of microglia to normal CNS development and homeostasis has been historically underappreciated. While microglia have long been recognized for their role as resident tissue macrophages, this extends considerably further than their innate immunological "first-line of defense" functions.

TABLE 1 | Methods of myeloid cell blockade and depletion.

Depletion method	Cell type targeted by method of depletion	Drawbacks	Mechanism of action
Intravenous administration of clodronate-encapsulated liposomes	Circulating monocytes and phagocytic cells in the bone marrow, liver and spleen (26–28)	Depletion is incomplete Clodronate liposomes do not specifically deplete one monocyte/macrophage subset	If clodronate is administered *via* an intravenous injection, clodronate liposomes in homeostatic animals cannot leave the blood vessels unless through sinusoids, and are thus limited to the circulation, bone marrow, liver, and spleen. In inflammatory conditions where the endothelium allows extravasation of molecules, liposomes can pass through. Liposomes containing clodronate are engulfed by phagocytic cells. Once in the cell, liposomes fuse with lysosomes causing the disruption of liposome bilayers, which allows the intracellular release of clodronate. Clodronate above a threshold concentration, causes irreversible damage to the cell and subsequent apoptosis (29).
Targeting chemokine receptor CCR2	CCR2-expressing monocytes in the bone marrow	Cells are not depleted but blocked from entering the circulation and thus do not reach inflamed tissue. Using anti-CCL2 (CCR2 ligand) monoclonal antibody (mAb) results in incomplete blockade (30).	Intravenous (i.v.) or intraperitoneal (i.p.) injection of anti-CCR2 or CCL2 mAb (31) or by the use of transgenic CCR2$^{-/-}$ mice (32). Monocytes are prevented from leaving the bone marrow via blocking the CCL2-CCR2 signaling axis.
Intracerebroventricular (i.c.v.) administration of clodronate liposomes	Microglia (33)	Invasive procedure which breaches the BBB Incomplete depletion	Clodronate liposomes are administered intracranially and engulfed by phagocytic cells in the brain, causing their "suicide" *via* apoptosis (26)
Transgenic animals with mutations in genes critical for microglial development and maintenance: PU.1, CD115 (CSF1R) and TGF-β	In CNS-TGFβ1$^{-/-}$ mice (*i.e.* IL2*TGF-β1*-Tg-TGF-β1$^{-/-}$: TGF-β1 is thus limited to T lymphocytes): Microglia (25) In CSF1R$^{-/-}$: Microglia, monocytes and tissue resident macrophages (5) In PU.1$^{-/-}$ mice: Microglia, mature myeloid cells and B cells (6)	These mice rarely survive into adulthood and develop defects in other organs other than the brain (34). Incomplete microglia depletion in CNS-TGFβ1$^{-/-}$ mice and an increase in peripherally derived cells into the CNS (CD39^{-}CD11b^{hi}Ly6C^{+}) (25)	Genes required for development and maintenance of microglia were genetically deleted, resulting in their depletion.
CD11b-HSVTK mice	Gamma-irradiation-resistant CD11b^{+} cells (*i.e.* microglia)	Incomplete bone marrow reconstitution and prolonged ganciclovir (GCV)-administration causes myelotoxicity and can be fatal (35). GCV administered orally or *via* an i.p. injection results in incomplete microglia depletion. Instead microglia proliferation and activation is blocked (35). Compromise of the BBB if GCV is administered i.c.v. and also extended application of GCV this way, causes microhemorrhages and influx of peripheral macrophages into the CNS (36).	Host mice express herpes-simplex virus thymidine kinase (HSV-TK) under the CD11b-promoter are lethally irradiated and engrafted with WT BM (35). Only irradiation resistant CD11b^{+} cells express HSV-TK. GCV administered *in-vivo* is converted into a monophosphorylated form *via* HSV-TK. Endogenous cellular kinases then convert the monophosphorylated form of GCV into a toxic triphosphate. GCV competes with thymine for DNA synthesis and thus preferentially targets proliferating cells. Non-proliferating cells have a reduced susceptibility to GCV. GCV administered orally or *via* an i.p. injection does not result in complete microglia depletion, but microglia "paralysis" whereby these cells are unable to proliferate or become "activated" (35). However, administrating GCV i.c.v. *via* an osmotic pump causes 90% depletion after two weeks (36).
CX3CR1^{CreER}DTR mice	Long lived CX3CR1^{+} cells (microglia, and most likely BAMs)	Repopulation in 5 days (23) Incomplete depletion—20% of microglia remained (23). Although Parkhurst et al. (37) showed a 99% depletion rate. Astrogliosis and "massive" production of cytokine and chemokines (cytokine storm) (23). Mice showed impaired learning and dendritic spine elimination (37)	Mice expressing Cre-recombinase (Cre-ER) under the *CX3CR1* promoter were crossed with iDTR animals. Tamoxifen (TAM) administration causes the nuclear translocation of the CreER fusion protein resulting in cre-mediated recombination and the expression of the diphtheria toxin receptor (DTR) on CX3CR1^{+} cells. Nuclear translocation of the CreER fusion protein is transient and lost shortly after TAM treatment in short-lived CX3CR1^{+} cells that are readily renewed in the BM *via* HSC. Long-lived CX3CR1^{+} cells express DTR, thus after systemic administration of diphtheria toxin (DTx), which can pass through the BBB, these cells are ablated. This system does not require the generation of a BM chimera and thus avoids the non-physiological effects observed

(Continued)

TABLE 1 | Continued

Depletion method	Cell type targeted by method of depletion	Drawbacks	Mechanism of action
			with whole body irradiation, including BBB disruption and peripheral immune cell infiltration into the CNS.
***Sall1*^{CreER}*Csf1r*$^{fl/fl}$ mice**	Microglia	Incomplete microglia ablation. 70–90% of microglia are deleted in various brain regions (38). Requires mouse breeding and generation of transgenic animals. Tamoxifen may result in an immunomodulatory phenotype in mice (34).	TAM administration induces the nuclear translocation of the CreER fusion protein in Sall1$^+$ cells. Cre-recombinase then drives the deletion of floxed *Csf1r*, causing the ablation of microglia (38). Sall1 is thought to be a microglia-specific marker, thus this depletion method is very specific to microglia.
Pharmacological inhibition of CD115 (CSF1R) using **PLX3397**	Microglia, HSC, osteoclasts, macrophages, and mast cells	Inhibits three other kinases including FLT3, PDGFR, and KIT (39–41). Repopulation once the drug is withdrawn Broad myelosuppression and astrogliosis (34).	PLX3397 is a CD115 (CSFIR) inhibitor that is typically formulating into a rodent chow and administered orally (19). CD115 signaling is required for microglial development and maintenance, thus inhibition of this receptor results in microglial ablation. Unlike all the other depletion methods listed above, microglia can be targeted without the breeding of transgenic animals, or the use of irradiation to achieve chimerism or the use of an invasive procedure which compromises the BBB. PLX3397, causes 50% microglia depletion within 3 days, and >99% depletion after 21 days of treatment (at 290 ppm) (19). At 75 ppm PLX3357 causes CSF1R inhibition without ablating microglia (21).
Pharmacological inhibition of CD115 (CSF1R) using **PLX5622**	Microglia	Rapid repopulation after the drug is withdrawn. Incomplete microglia depletion (21) Affects haemopoiesis and macrophage phenotype and function in the spleen, BM and blood (42–44)	PLX5622, like PLX3397 is a CD115 (CSFIR) inhibitor which is also typically formulated into a rodent chow to be administered orally. Both PLX3397 and PLX5622 have the same potency for inhibiting CD115. PLX5622, however, has a 20-fold selectivity for CD115 over other kinases (KIT and FLT3) and a ~15% increase in BBB penetrance (has a lower molecular weight, higher lipophilicity, and better cell permeability), compared to PLX3397 (21) and can yield 90% microglia depletion within 5 days (at 1,200 ppm in chow).

Embryonic Brain

As microglia seed the brain during early embryogenesis, they display an "activated," ameboid morphology as they proliferate and migrate throughout the CNS (48, 49). Upon CNS maturation, microglia become more sessile and adopt a highly ramified morphology (49). The importance of microglia to embryonic development in the CNS has been shown in several depletion models, with ablation of these cells causing long term effects on normal brain functioning. For example, the absence of embryonic microglial progenitors caused defects in dopamine innervation and cortical networks (50), whilst neuronal survival was reduced in CX3CR1-deficient and microglia-ablated CD11b-DTR mice, arguably from the absence of CX3CR1-dependent production of neurotrophic insulin-like growth factor-1 (IGF-1) (51). Absence of microglia in mice homozygous for the null mutation in the CSF-1 receptor (*Csf1r*$^{-/-}$) revealed a disruption to brain morphology and neuronal density, as well as significantly affecting total astrocyte and oligodendrocyte numbers (52). Further, depletion of microglia using PLX5622 resulted in sex-specific behavior effects, with female mice developing long-term hyperactivity and anxiolytic-like behavior (46).

In the developing brain, microglia shape neural circuitry by: 1) inducing neuronal cell death *via* the release of superoxide ions (53, 54), 2) clearing viable (55) and apoptotic neural progenitors (56), 3) promoting neurogenesis *via* the release of IL-1β, IL-6, TNF, and IFN-γ (57–60), and 4) paring down supernumerary synapses, whilst strengthening functional ones (61–63). A number of mechanisms have been identified which contribute to microglial-meditated synapse modulation. Complement cascade components, C1q and C3, localized to neuronal synapses, promote microglial synapse engulfment (60, 64), while CD47 localized to neurons provides a "don't eat me signal" to microglia that express CD172a (SIRPα), thereby preventing aberrant synaptic phagocytosis (65). Serotonin signaling (66), triggering receptor expressed on myeloid cells 2 (TREM2)-dependent functions (67), the CX3CR1-CX3CL1 axis (62) and microglial interaction with neuronal-expressed major histocompatibility complex (MHC) class I (68–71) are also thought to be involved in microglial-mediated synapse elimination. Microglia express CX3CR1 (62), TREM2 (67), and a serotonin receptor (5-HT2B) (66), with the latter enabling their movement towards serotonin. Knockout of these receptors results in defects in synaptic refinement (CX3CR1 and TREM2) or the organization of retinal projections (5-HT2B). Although microglia can prune superfluous synapses, they can also promote the formation of new ones (37, 72).

Beyond shaping neuronal circuitry, microglia are also required for vascularization, myelination, and gliogenesis. Microglia are recruited to growing vessels to promote vascular network formation in the retina (73, 74) and this is *via* release of angiogenic factors other than vascular endothelial growth factor–A (74). CD11c$^+$ microglia, which expand in the postnatal brain, express a neurosupportive gene signature and IGF-1 and are required for myelinogenesis during development (75). More

recently, a new role in gliogenesis has been identified for microglia at the later embryonic stages of E15.5 and E17.5 (76). A subpopulation of amoeboid microglia lining the tuberal hypothalamic third ventricle have been found to influence glial precursors *via* chemokine signaling, namely, CCL2 and CXCL10, which are required for the migration and maturation of oligodendrocytes, but not astrocytes (76). An additional unique microglial subset (*i.e.* proliferative-region associated microglia or PAM) enriched in metabolic genes and found in the first postnatal week in the corpus callosum and cerebellar white matter were found to be specialized in the clearance of newly formed oligodendrocytes (77).

Adult Brain

In the adult brain, microglia tile the parenchyma in a grid-like fashion, displaying a ramified morphology with static somata and "never-resting" cytoplasmic extensions (78). These extensions survey the CNS microenvironment using their "sensome" to identify and respond to perturbations that may threaten homeostasis (79). The TWIK-related Halothane-Inhibited K^+ channel, a tonically active potassium channel expressed by microglia, regulates the ramification and movement of microglial processes to support homeostatic surveillance of CNS activity (80). The microglial "sensome" comprises microglia-expressed genes encoding receptors and signaling molecules that enable detection of pathogen invasion, cytokines, pH alterations, metabolites, ATP, and adenosine. These include toll-like receptors (*Tlr2* and *Tlr7*), chemokine receptors (*Ccr5*, *Cx3cr1*, *Cxcr4*, and *Cxcr2*), Interferon-induced transmembrane proteins (*Ifitm2*, *Ifitm3*, and *Ifitm6*), Fc receptors (*Fcer1g* and *Fcgr3*), siglecs (*SiglecH* and *Siglec3/Cd33*), and purinergic receptors (*P2rx4*, *P2rx7*, *P2ry12*, *P2ry13*, and *P2ry6*) (79). P2RY12 and SiglecH are microglia-specific in the CNS, with P2RY12 importantly involved in chemotaxis towards neuronal and CNS damage *via* the detection of ATP or ADP (81, 82). In the aging brain, 81% of these genes are downregulated, with some genes, including *Cxcr4*, *Cxcr2*, *Tlr2*, *Ifitm2*, *Ifitm3*, *Ifitm6*, and *P2rx4*, being upregulated (79). This is thought to contribute to age-related microglial neurotoxicity (79) and potentially reduced microglial phagocytic activity that occurs with aging (83). Microglia also display an increased expression of CD11b, MHC-II, CD68, and CD86 proteins and expression of *Tnf*, *Il-6*, and *Il-1β* RNA in the aging brain, collectively suggesting an enhanced inflammatory profile and reduced homeostatic function with age (84).

The maintenance of a surveillant microglial state under physiological conditions is ultimately likely to be a vectorial outcome of a number of signals, including neuronal and astrocyte-derived factors, microglia-expressed CX3CR1, CD200 receptor (CD200R), and CD172a, which dampen microglial activity through binding their respective ligands, CX3CL1 (expressed by neurons), CD200 (expressed by neurons, astrocytes, and oligodendrocytes), and CD47 (expressed ubiquitously, including on neurons) (85), as well as through increased expression of microRNA-124 (86) and TGF-β signaling (25).

Besides tissue surveillance, microglia are involved in synapse formation and learning in the adult CNS *via* the secretion of brain-derived neurotropic factor (37). Microglia are also required for synaptic pruning, with the purine receptor P2RY12 important for synaptic plasticity in the visual cortex of the adolescent CNS (87). In contrast, the CX3CR1-CX3CL1 (62) and CR3/CD11b (60) axis appear to be more critical during development for microglial-mediated synaptic pruning. Microglia also support adult neurogenesis, with a unique population of microglia expressing low levels of purine receptors in the subventricular zone and rostral migratory stream required for survival and migration of newly generated neuroblasts (88).

The role of microglia as phagocytes also plays a major part in homeostasis, enabling clearance of debris, apoptotic, and surplus cells (89) to maintain optimal neural function. Microglial-expressed TAM receptor kinases, MER Proto-Oncogene Tyrosine Kinase (MerTK), and Axl have revealed an important role for neuronal progenitor cell clearance (90), which may be required for efficient neurogenesis, whereas CD11b, TREM2, TIM-4, and BAI1 appear to be required for the phagocytosis of apoptotic neurons (53, 91, 92). Microglia can recognize a number of "eat-me" signals, including phosphatidylserine, components of the complement system, thrombospondin and uridine 5′-diphosphate (93), which stimulate phagocytosis (68, 76, 93). Although microglia are the principle phagocytes in the CNS, other glia, including oligodendrocytes and astrocytes, are also thought to contribute to this function (94, 95).

MICROGLIAL "ACTIVATION"

Microglial "activation" refers to a reversible, transient state, defined by a morphological and functional phenotype distinct from homeostatic microglia. Before the advent of *in vivo* imaging, microglia in steady state homeostasis were classified as "resting." However, it is now clear that although the cell soma may remain in one site, the processes of each microglia continuously explore the microenvironment in a highly dynamic manner (78).

In the steady state, microglia have a small cell soma with long, thin hyper-ramified cytoplasmic processes. On detection of a noxious signal (toxins, pathogens, endogenous proteins) or neuronal damage, microglia undergo a rapid morphological transition, retracting their processes to become shorter and thicker, acquiring a more ameboid morphology, and undergoing hypertrophy, thus increasing their somatic surface area. In addition to these morphological adaptations, often referred to as microgliosis, microglia undergo transcriptional and phenotypic changes in a context-dependent manner. This reactive phenotype is associated with changes to motile, proliferative, and phagocytic functions (96, 97) and invites comparison with microglia that populate the early CNS. Historically, alterations in microglial morphology and/or the upregulation of CD45, Iba1, Griffonia *simplicifolia*-lectin, and MHC-II were the first reliable indicators of microglial "activation" that implicated microglia in CNS pathology.

Intermediate morphological activated states of microglia have also been identified, which are described as "rod-like," "hyper-ramified," "bi-polar," and "bushy" (98). However, it is clear that

microglial form and function do not necessarily correspond, as microglia are observed to display both classic "resting" and "activated" morphologies in human CNS inflammation and neurological and psychiatric disease (99). Despite morphological measurements (cell somatic area, dendrite length and number, total cell area, and parenchymal cell density) being the primary technique used to study these cells for decades, there are no standard parameters that link these forms to function and more detailed *in situ* molecular and protein profiling techniques, paired with imaging will be required to fill this gap. The Hyperion is an imaging mass cytometer and one of the first multiplexed imaging technologies developed which theoretically enables the detection of >100 different metal-conjugated markers (currently 49) to enable spatial resolution of protein expression in tissue sections (100). Other competing high-dimensional imaging systems include the CODEX, GeoMx DSP, and the MACSima by Akoya Biosciences, Nanostring, and Miltenyi Biotec, respectively. To fully recapitulate the dynamic nature of these cells in tissues in real-time, *in vivo* imaging techniques, such as intravital microscopy (IVM) can be employed. However, with the limited number of fluorescent probes and mouse models available for IVM, correlative imaging, combining data from fluorescence, light and electron microscopic modalities provide additional structure-function information (101, 102).

Advances in high-dimensional and single-cell molecular and immune profiling technologies have effectively invalidated classical microglial characterization approaches. The descriptive "resting" *versus* "activated" and "M1" *versus* "M2" nomenclature oversimplified microglial behavior, suggesting they exhibited dichotomous "yin-yang"-like functions. These concepts have been rejected by the field (103) and are being replaced by multi-dimensional activation states, in which function is programmed and then finely tuned according to the prevailing microenvironment, in a context-, sex-, region-, developmental-, disease-, and even disease stage-specific manner. It is still accepted that microglia have pro-inflammatory ("M1") and anti-inflammatory ("M2") functions, but these are now understood to co-exist, with microglia capable of co-expressing M1-like and M2-like markers in a context-dependent manner. Thus "disease-associated" microglia (DAMs) in a mouse model of Alzheimer's disease (104), "microglial neurodegenerative" phenotype (MGnD) in mouse models of AD and ALS (105) and disease-associated microglia (daMG1-4) in experimental autoimmune encephalomyelitis (EAE) (106) are superseding earlier and more simplistic terms, to incorporate the idea that microglia can have unique molecular and/or immunological profiles and/or functions in different disease contexts.

ORIGIN AND CLASSIFICATION OF MONOCYTES AND MONOCYTE-DERIVED CELLS

During certain diseases and/or injuries involving breach of the BBB, BM-derived monocytes infiltrate the CNS parenchyma and intermingle with the resident microglial population. Despite often close phenotypic similarities, these infiltrating myeloid cells are developmentally distinct from microglia and give rise to effector cells whose functions are presumably not fulfilled by their resident counterparts. In contrast to the YS-origin of microglia, monocytes are hematopoietic cells that originate in the BM. In adulthood, these cells are derived from definitive HSC and mature from monocyte-dendritic cell (MDP) precursors, common monocyte progenitors (cMoP), and granulocyte and macrophage progenitors (GMP) through a series of sequential differentiation steps in the BM (107, 108). The fate of these monocytes is specified by the expression of transcription factors PU.1, IRF8, Klf4, and GATA2 (3, 109–111), and their differentiation, survival, and proliferation is regulated by the growth factor receptor CD115 and its ligand M-CSF (112–114). Following their generation in the BM, monocytes are released into the peripheral circulation.

Circulating monocytes are composed of multiple subsets that differ in their phenotype, size, transcriptional profiles, and migratory properties. These distinct monocyte subsets are characterized by their differential expression of CD14 and CD16 in humans (115) and by the surface marker combination Ly6C, CD62L, CD43, and the chemokine receptors CX3CR1 and C-C chemokine receptor 2 (CCR2) in mice (116) (**Figure 1**). In humans, 80–90% of the monocyte pool is composed of $CD14^{+}CD16^{-}$ classical monocytes with the remaining 10–20% shared by $CD14^{+}CD16^{+}$ intermediate and $CD14^{lo}CD16^{+}$ non-classical monocytes (115). The generation of a mouse strain in which a GFP reporter was engineered into the *CX3CR1* locus ($CX3CR1^{GFP}$ mice) (117) enabled the discovery of two corresponding monocyte subsets (116). In mice, "classical" monocytes (also known as "inflammatory monocytes") are characterized by their expression of surface markers $Ly6C^{hi}$, $CX3CR1^{int,}$ $CCR2^{+}$, $CD62L^{+}$, and $CD43^{lo}$, whereas "non-classical" monocytes (also referred to as "patrolling monocytes") are defined as $Ly6C^{lo}$, $CX3CR1^{hi}$, $CCR2^{lo}$, $CD62L^{-}$, and $CD43^{+}$ cells (116, 118, 119). Transcriptional comparison between mouse and human monocyte subsets correlated $Ly6C^{hi}$ monocytes with classical $CD14^{+}CD16^{-}$ monocytes and $Ly6C^{lo}$ monocytes with non-classical $CD14^{lo}CD16^{+}$ monocytes (120).

As a component of the mononuclear phagocyte system, circulating monocytes were historically considered to be the definitive precursors of tissue-resident macrophages and dendritic cells (DC) (121). However, recent studies have demonstrated that most tissue-resident macrophages are of embryonic origin (122, 123), although conventional DCs have a distinct BM precursor (124). Today, monocytes are viewed as a distinctive cell type with diverse functions. In the steady state, $Ly6C^{+}$ monocytes can traffic to various tissues and maintain their monocytic transcriptional profile (119), but they can also give rise to a proportion of tissue-resident myeloid cells (123) or transition into $Ly6C^{lo}$ monocytes (123, 125, 126). During inflammation, monocytes can give rise to macrophages (monocyte-derived macrophages or MDMs) and DCs (monocyte-derived DC or moDCs) with non-redundant

functions that often cannot be fulfilled by their resident counterparts (126). Collectively, these distinctive cell types have been classified according to their monocytic origin as "monocyte-derived cells" (MDC) (127) (**Figure 1**).

Under homeostatic conditions, Ly6C^{hi} monocyte progeny are present in almost all tissues, where they constitute a minor fraction of the tissue-resident macrophage pool (119, 128–134). The CNS parenchyma is a notable exception, where little to no monocyte immigration is observed in the steady state (5, 12, 135), although a proportion of choroid plexus and dural macrophages are evidently replenished by BM-derived monocytes during homeostasis (15, 136).

ONTOGENY AND DIFFERENTIATION OF MONOCYTE-DERIVED CELLS IN THE INFLAMED CNS

In contrast to homeostasis, during inflammation Ly6C^{hi} monocytes may rapidly infiltrate the diseased CNS, usually in a CCR2-dependent manner. This may be facilitated by compromise of the BBB, but not necessarily (30, 137). Although monocyte recruitment and infiltration is well described in the acutely diseased brain, the behavior of these cells is more controversial in chronic, low grade inflammation observed in aging and stress. Thus, despite increased BBB permeability with age, monocyte infiltration does not inevitably accompany healthy aging (138). On the other hand, inflammation associated with psychosocial stress may promote monocyte infiltration into the CNS (139, 140), although this has been contested (141, 142).

Interactions between monocytes and CNS borders critically affect their recruitment, infiltration, and differentiation during neuroinflammation. The different ports of entry into the CNS have been implicated in shaping either a protective or pathogenic monocyte response. For instance, the differential expression of CX3CR1 and CCR2 ligands may selectively recruit either "pro-inflammatory" (Ly6C^{hi}CCR2^{+}) or "pro-resolution" (Ly6C^{lo}CX3CR1^{hi}) monocyte-derived cells. This is supported by experiments showing that Ly6C^{lo}CX3CR1^{hi} monocytes, which aid recovery from spinal cord injury, entered the CNS *via* the choroid plexus and migrated to the injury site through the central canal in an α4-integrin/vascular cell adhesion molecule-1- and CD73-dependent manner. In contrast, Ly6C^{hi}CCR2^{+} pro-inflammatory monocytes entered the CNS *via* the parenchymal blood vasculature in a CCL2-dependent manner and mediated secondary injury (143). Although Ly6C^{hi} and Ly6C^{lo} monocytes are thought to be independently recruited to the CNS, the transition of Ly6C^{hi} monocytes to Ly6C^{lo} monocytes has been observed during both homeostasis and inflammation, and the recruitment of Ly6C^{lo} monocytes is at least partially CCR2-dependent (123, 125, 126). It is possible that the transition from Ly6C^{hi} to Ly6C^{lo} monocytes is influenced by different CNS entry points, such that monocytes traversing through choroid plexus and leptomeninges encounter stimuli driving their differentiation into Ly6C^{lo} monocytes, whereas those traversing through the parenchymal vasculature remain undifferentiated inflammatory monocytes. Alternatively, the endothelium may better enable the emigration of Ly6C^{hi} cells from the CNS parenchymal vasculature (144). Future studies investigating how endogenous macrophages and/or endothelium at various CNS-entry points may shape the phenotypic and functional profiles of CNS-infiltrating Ly6C^{hi} monocytes in the mature animal are needed to address these gaps. Furthermore, what changes occur during development of the BBB that enable differential diapedesis during maturation of the adaptive immune system have yet to be fully elucidated.

Once in the CNS parenchyma, local microenvironmental cues can shape MDMs to adopt a phenotype similar to those of CNS-resident macrophages. Using CCR2-red fluorescent reporter (RFP) mice, a recent study found CNS-infiltrating CCR2^{+}CD206^{+} monocyte-derived cells localized beside CCR2^{-}CD206^{+} resident macrophages in the leptomeninges and perivascular space, demonstrating these cells can gain phenotypic markers characteristic of CNS-resident myeloid cells (106). Similarly, CNS-infiltrating monocytes adopt a phenotype indistinguishable from microglia in the acute phase of EAE, although these cells do not appear to integrate into the CNS-resident microglia population following the resolution of inflammation (145, 146).

Emergency conditions may additionally generate ontogenically distinct monocyte subsets whose presence is restricted to inflammatory conditions. As severe inflammation requires the constant generation and mobilization of monocytes to the inflamed brain, emergency monopoiesis can generate GMP-, MDP-, and cMoP-derived monocytes that appear under inflammatory conditions (108) and may perhaps bypass the canonical Ly6C^{+} monocyte intermediate (147). In the inflamed brain, such populations may include *Cxcl10*$^{+}$ and *Saa3*$^{+}$ monocytes, the former having been identified in EAE and possibly cerebral malaria (147, 148). Whether these "emergency" monocyte populations are functionally distinct from Ly6C^{hi} monocyte-derived cells is unclear, although recent evidence suggests these subsets may differentially contribute to pathology (147). Further fate-mapping and functional studies investigating emergency monocyte populations in the inflamed CNS will be needed to assess whether these cells are ontogenically and functionally distinct from those derived from Ly6C^{hi} monocytes during neuroinflammation. Taken together, monocytes represent a particular unique, plastic cell type equipped with a diverse differential program that enables their context-dependent effector functions upon entry into the CNS.

IDENTIFYING MICROGLIA IN THE HOMOEOSTATIC AND INFLAMED BRAIN

Studying microglial behavior in the brain is difficult, even under homeostatic conditions. Separating microglial functions from other neuroglial or peripherally derived immune cell responses is challenged firstly by the difficulty of culturing adult murine

microglia (149) and secondly, by their tendency to alter their transcriptome *ex vivo*. Human and mouse microglia lose their *in vivo* transcriptional profile upon isolation, with significant differences in mRNA signatures between recently isolated microglia and *in vitro*-cultured microglia (25, 150), although the *in vivo* profile may be restored when cells are put back into an intact brain (151). This emphasizes the likely need for interaction with other CNS cell types for "normalcy" and it is likely that loss of environmental cues remodel the regulatory milieu *in vitro*, inducing substantial changes in microglial gene expression (150). Culturing mouse and human microglia for only 6 h induced upregulation of genes related to acute inflammatory response and stress and downregulation of genes associated with immune functions, as well as blood vessel and brain development (150). Although culturing conditions required to maintain the *in vivo* microglial transcriptome are unknown, brain-specific signals are almost certainly required, currently limiting the interpretation of *in vitro* observations. Our growing understanding of the inextricable importance of the brain microenvironment in instructing microglial phenotype and behavior thus drives an increasing emphasis on work *in vivo*.

In the homeostatic brain, microglia are easily identifiable from other cells in the CNS (see **Table 2** for a list of microglial phenotypes identified in the adult murine brain in steady state). Microglia comprise the largest myeloid population in the CNS and can be identified using imaging or single-cell cytometry systems with one or two of a wide range of phenotypic and/or functional markers, *e.g.*, CD45, CX3CR1, CD11b, F4/80, CD64, CD68, transmembrane protein 119 (TMEM119), purinergic receptor P2Y, G-protein-coupled 12 (P2RY12), CD115 (CSF-1R), CD200R, CD172a (SIRPα), CD317, MerTK, 4D4, lymphocyte antigen 86 (LY86), secreted protein acidic and rich in cysteine (SPARC), CD162, and Fc receptor-like S (FCRLS) (106, 136, 146, 153–157) (**Figure 1** and **Table 2**). Using flow, mass, and spectral cytometry, murine and human homeostatic microglia are typically identified as $CD45^{lo}CD11b^{+}$ (30, 154, 158). Non-parenchymal brain macrophages, *i.e.*, dural, meningeal, perivascular, and choroid plexus macrophages, collectively called CNS- or border-associated macrophages (CAMS or BAMS) (159–161), have a higher expression of CD45 ($CD11b^{+}CD45^{int}$) and/or do not express microglia-specific markers, making these cells distinguishable from microglia (136). By immunohistochemical techniques, microglia are commonly recognized by their immunoreactivity to Iba1, CD11b, CD68, and GS-lectin. Moreover, the highly ramified morphology of microglia makes them readily distinguishable from other myeloid cells in the brain, which are more amoeboid in shape (162).

However, identification of microglia using immunohistochemistry or cytometry becomes increasingly complicated during neuroinflammation with the infiltration of BM-derived monocytes that adopt a phenotype and morphology similar to reactive microglia. Infiltration of MDMs into the CNS is a hallmark of a number of acute and chronic neuropathologies, including autoimmunity, neurodegeneration, stroke, traumatic injury, and infection, with each disease context associated with a varying degree of CNS infiltration, inflammation, as well as differential MDM and microglial phenotype and function.

CNS-infiltrating MDMs express molecular markers common to microglia, including CX3CR1, CD11b, F4/80, CD45, CD64, CD115, and Iba1, to name a few (154). On the other hand, these cells express higher amounts of Ly6C, CD44, CD45, CD49d, CD11a, CXCR4, and CCR2 and have a lower expression of CX3CR1 (30, 153, 154, 163–165). These markers, however, can be downregulated over the course of disease. Typically, MDMs are identified as $CD11b^{+}CD45^{hi}$. However, since BAMs are also $CD45^{int/hi}$ and "activated" microglia upregulate CD45, this gating system fails to accurately discriminate between these cells. This is particularly true in severe inflammatory conditions, such as West Nile virus (WNV) encephalitis, where there is substantial and sustained infiltration of MDMs into the CNS (30). Thus, the ability to resolve populations during neuroinflammation has historically been impossible without

TABLE 2 | Genes and proteins expressed by microglia in steady state.

Transcriptome	**Two microglia subsets, hMG1 and hMG2, both expressing**: *Bhlhe41*, *Gpr34*, *Hexb*, *Olfml3*, *P2ry12*, *P2ry13*, *Sall1*, *Serpine2*, *Siglech*, *Sparc*, *Cx3cr1*, *Fcr1*, *Csfr*, *Csf1*, *C1qc*, *C1qb*, *C1qa*, *Tmem119*, *Trem2*, and *Slc2a5* (hMG1 express genes related to the ERK1 and ERK2 cascade as well as responses to IFN-γ) [Single-cell RNAseq, (106)]
	Hexb, *Cst3*, *Cx3cr1*, *Ctsd*, *Csf1r*, *Ctss*, *Sparc*, *Tmsb4x*, *P2ry12*, *C1qa*, and *C1qb* [Single-cell RNAseq, (104)]
	Fcrls, *Trem2*, *Hexb*, *Olfml3*, *Gpr34*, *Tmem119*, *P2ry12*, *Siglech*, *Golm1*, *Sall1*, *Adgrg1*, *Slc2a5*, *Serpine2*, *Sparc*, *Adamts1*, *Itgam*, *Aif1*, *Cx3cr1*, *Csf1r*, *Cd68*, *Adgre1*, *Fcgr1*, and *MerTK* [Single-cell RNAseq (136)]
Proteome	$CD45^{+}CD11b^{+}F4/80^{+}CD64^{+}MerTK^{+}CD24^{+}$ $CD172a^{+}$ [CyTOF (152)].
	Two microglia subsets, A and B, both expressing: $CD45^{+}CD11b^{+}CD317^{+}MHC\text{-}II^{-}$ $CD88^{+}MHCI^{+}MerTK^{+}4D4^{+}FCRLS^{+}$ ***Unique expression profiles between the microglia subsets:*** **Pop A:** $CD39^{low}CD86^{-}$ **Pop B:** $CD39^{hi}CD86^{+}$ [CYTOF (146)]
	$CD162^{+}P2RY12^{+}TMEM119^{+}Ly86^{+}Iba\text{-}1^{+}$ $SPARC^{+}$ [IHC (106)]
	Three microglia subsets, 1–3, all expressing: $CD45^{low}CX3CR1^{+}CD11b^{+}F4/80^{low/-}$ ***Unique expression profiles between the three microglia subsets:*** **Subset 1:** $CD14^{+}TCR\text{-}\beta^{+}$ **Subset 2:** $CXCR4^{+}CCR5^{+}CD115^{+}$ (Could represent a more motile population) **Subset 3:** $MHCII^{+}$ (Could be of peripheral origin) [CyTOF (153)]a
	$CD45^{+}CD64^{hi}CD11c^{low}MMR^{low}MHCII^{low}CD11b^{hi}CLEC12A^{low}$ $NRP1^{low}CD63^{low}$[Flow cytometry (136)]

recourse to adoptive transfers, parabiosis, or chimeric animals made by lethal gamma-irradiation and BM reconstitution. Although identification of resident and infiltrating cells becomes clearer using such techniques, the non-physiological conditions may confound the accurate interpretation of results.

TOOLS USED TO DISCRIMINATE RESIDENT AND INFILTRATING MYELOID CELLS IN THE INFLAMED BRAIN

Recent advances in single-cell sequencing technologies has shed light on some uniquely expressed microglial genes including *Fcrls*, *P2ry12* (25), *Spalt-like transcription factor 1* (*SALL1*) (38), *sialic acid-binding immunoglobulin-type lectin H* (*Siglec-H*), and *Tmem119* (166). The development of RNA primers and antibodies against these "microglia-specific" markers have substantially aided in the resolution of myeloid populations in the CNS, without the need for more complicated experimental manipulation. Transgenic animals expressing fluorescent reporters that identify microglia, or Cre-recombinase and/or HSV-TK under "microglia-specific" promoters that can be used to deplete microglia, have also been an important advance on the use of CX3CR1 or CD11b promoters, which also act on myeloid cells in the periphery.

However, the discovery that microglia-specific markers P2RY12 and TMEM119 are downregulated in neurodegeneration and neuroinflammation (105, 106, 167), has reduced their value for identification of microglia in such models. Nonetheless, the expression of these markers appears to be model-dependent and therefore more useful in specific diseased-states. P2RY12 was upregulated in models of pseudorabies virus encephalitis (168) and neuropathic pain (169), whilst P2RY12 (170) and TMEM119 (163, 171) were stably expressed during stroke. However, both markers have been shown to be expressed by peripherally-derived myeloid cells in the CNS (9, 163), with TMEM119 also expressed by other non-CNS cell types (172). TMEM119, originally shown to be expressed in mouse osteoblasts, is additionally expressed in human bone tissue, DCs, osteosarcoma, and lymphoid tissue (173, 174). FCRLS, also previously thought to be microglia-specific, has been observed in all CNS-associated macrophage subsets (106). Notwithstanding these limitations, these markers are still specific for microglia in the homeostatic CNS and will likely remain important tools for elucidating function.

Another major advance in microglial biology has been the discovery of PLX5622 (Plexxikon Inc.) (21), a small molecule CD115 inhibitor that penetrates the BBB and depletes microglia in as little as three days (175, 176) (**Table 1**). Other studies have reported near to complete microglial depletion within 7, 14, or 21 days. Not surprisingly, other cells dependent on CD115 signaling are also modulated by PLX5622 treatment, including lymphocytes and myeloid cells in the spleen, blood and BM (42). Moreover, some microglia are resistant to depletion even after prolonged treatment, making this approach unsuitable for studying all microglia subtypes (21). Despite these limitations, PLX5622 is a major improvement from previously used depletion methods including i.c.v.-injected clodronate liposomes, PLX3397 (also a CD115 inhibitor), CD11b-HSVTK, and *CX3CR1*^{CreER}DTR mice, all of which may non-specifically target other leukocytes, with some methods taking longer for microglial ablation to occur or associated with incomplete microglial depletion and/or toxicity, off-target effects, or BBB damage (34) (**Table 1**). Moreover, PLX5622, unlike PLX3397, has a 20-fold greater selectivity for CD115 than for other kinases, as well as increased BBB penetration (21).

Although PLX5622 has become the gold standard microglial depletion method, CNS changes that subsequently occur in the absence of microglia and/or in the presence of dead microglia, limit the accurate interpretation of their cellular functions *in vivo*. *In vivo* fate-mapping models used to track peripheral or resident cells have largely overcome this limitation. The development of site-specific recombinases and transgenic mice, for instance, have provided tools to genetically mark cell lineages and their descendants, enabling the mapping of cell interaction and migration, lineage segregation and proliferation (177–179). Thus, unlike the aforementioned methodologies used to study microglial functions, fate-mapping provides a targeted and non-invasive approach that can be used during development and adulthood. Further, in contrast to conventional reporter strains whereby mice express fluorescent reporters under specific promoters (e.g. CX3CR1$^{GFP/+}$ or CCR2 $^{GFP/+}$ or CX3CR1$^{GFP/+}$; CCR2 $^{GFP/+}$ mice), fate-mapping does not require markers to be stably expressed by cells. Thus, enabling the identification of cells following the downregulation of relevant genetic markers. Fate-mapping approaches have been used in a number of neuroinflammatory models to distinguish resident from infiltrating myeloid cells (9, 163). For example, using *Cxcr4*$^{CreER/Wt}$; *R26*$^{CAG\text{-}LSL\text{-}tdT}$ mice in a stroke model, HSC-derived myeloid cells were traceable by tdTomato (tdT) fluorescence (163). Moreover, the ubiquitously active CAG promoter in *R26*$^{CAG\text{-}LSL\text{-}tdT}$ enabled MDMs to be traced, despite their downregulation of CXCR4 in the CNS during stroke. A similar approach was used in neonatal stroke and development using bi-transgenic CCR2-CreER$^{tg/+}$; R26R-EGFP$^{tg/+}$ mice, where Ly6C^{hi} and Ly6C^{lo} cells could be mapped despite downregulation of CCR2 (9). Although fate-mapping is a powerful approach that can be used to study microglial functions *in-vivo*, these models can be time-consuming and costly to generate, as well as requiring cell-specific markers to target particular cell types.

The development of high-parameter cytometry systems, including mass and spectral cytometry have further aided the necessary discrimination of populations without genetic manipulation. With a generally enhanced signal sensitivity, spectral cytometers such as the Cytek® Aurora can enable more accurate separation of cells which may differ in their relative expression of single and/or dim markers. The ability to measure a greater number of fluorescent signals in one assay and the speed of acquisition gives spectral cytometry a significant advantage over conventional fluorescence flow and mass

cytometry (180). Nevertheless, high-dimensional immune profiling by these modalities, in conjunction with dimensionality-reduction algorithms, such as t-distributed stochastic neighbor embedding (tSNE) and uniform manifold approximation (UMAP), which enable the visualization of high-dimensional data on a 2D plot, provides important tools for more detailed population identification and separation (181–184). The use of unbiased clustering and dimensionality-reduction approaches assist in the identification of subpopulations with a range of differentially expressed markers. The development of novel gating strategies arising from this separation further enable cell types to be sorted for more detailed *in vitro* or *in vivo* functional or RNA analysis. In EAE, for instance, three microglial subpopulations were identified with mass cytometry by two independent groups (106, 146), with one of these studies also identifying five MDM subsets (146). Understanding the protective or pathogenic functions of these cell types will inform targeted cell-specific therapies in these diseases. Taken together, the development of new tools to resolve myeloid populations in the CNS has substantially enhanced our understanding of their functions and heterogeneity in health and disease.

SHOES TOO BIG TO FILL? CAN MONOCYTE-DERIVED MACROPHAGES ACQUIRE A MICROGLIAL IDENTITY?

Considering microglia seed the brain during early embryogenesis, where they participate in CNS development, support neuronal networks, and adopt memory-like functions as they persist throughout adulthood, is it possible for MDMs, with a different origin, epigenome, and transcriptome, to acquire a "true" or even a functional microglial identity? Similar to microglia, tissue-resident Kupffer cells in the liver and alveolar cells in the lung are established before birth and are subsequently renewed in situ independently of BM-derived monocytes (7, 132). However, monocytes show minimal transcriptomic differences with their embryonic counterparts and can differentiate into both Kupffer cells and alveolar macrophages (185–187), but evidently not into microglia. Peripheral monocytes can populate the CNS, but they differ phenotypically, have a non-redundant role and a different molecular signature from embryonically seeded microglia (25, 188). Even after prolonged engraftment in the brain, MDM responses to lipopolysaccharide challenge, chromatin landscapes and ~2000 transcripts remained different from resident microglia (189). Engrafted MDMs did, however, adopt other microglial characteristics including self-renewal, resistance to γ-irradiation and a ramified morphology (190). In contrast, donor microglial cells fully adopt the transcriptomic identity of embryonically derived microglia in microglia-deficient CD115 knockout mice (191). Why BM-derived myeloid cells only become "microglia-like" in the CNS is currently unknown, but the EMP origin of microglia and the unique CNS tissue microenvironment likely plays a critical role (150, 191).

In contrast, a small population of microglia are reported to be derived from BM-derived HSCs during embryogenesis, suggesting a monocyte to microglia switch (9). This has also been demonstrated during neonatal stroke using a fate-mapping model, where invading monocytes became DCs or microglia-like cells (9). Microglia-like cells were present 62 days post-stroke, with many exhibiting a ramified morphology, P2RY12 and TMEM119 immunopositivity and expression of *Sall1* mRNA. In another stroke model, MDMs ectopically placed in the peri infarct region of *Cxcr4* knockout mice became positive for P2RY12 and TMEM119 (163). In WNV encephalitis models, $Ly6C^{hi}$ monocytes migrate from the BM to the CNS, where they assume a phenotype indistinguishable from activated microglia, with regard to CD45 and CD11b expression (30, 192). Contrary to the view that microglia-like cells enter the brain only when the BBB is perturbed, the BBB is only sporadically affected in this model (30). Some of these peripherally derived monocytes also became ramified in the parenchyma of the brain (30).

Further investigation is required to understand why infiltrating MDMs express microglial molecules in the CNS *de novo*, and the putative functions of these peripherally derived cells, relative to their resident counterparts. It is possible that TMEM119 and P2RY12 are not microglia-specific in the inflamed CNS, or that the inflammatory milieu in stroke, coupled with the prolonged time MDMs spend in the CNS, enables them to acquire a microglia-like phenotype, particularly as the CNS microenvironment ordinarily defines microglial phenotype and identity (150, 191). The degree of inflammation may be important; WNV causes a fatal encephalitis characterized by severe inflammatory monocyte infiltration that involves the entire CNS (30), whereas models such as EAE or AD, used to investigate microglial activity, are accompanied only by localized foci of inflammation and/or much less severe inflammation overall. As such, the response observed in WNV may be in stark contrast to what has previously been described. It is worth reflecting from an evolutionary point of view that the biggest threat to survival is infection, against which the best defense is the primed innate and adaptive immune systems. Long-lived animals are subject to many infections over a lifetime, as well as having an environmentally increased probability of being infected by the same pathogen more than once. As such, it seems reasonable that myeloid reservoirs in the BM compartment could be recruited to the brain to perform microglial functions in the interim. Setting up novel "microglial" networks during a first CNS infection in a high prevalence environment, despite a possible functional cost, may be a useful survival strategy for effective early CNS defense by the innate immune response in the event of novel or recurring future infections. Irrespective of whether MDMs can become microglia physiologically, current approaches are being developed with the intention of engineering these cells for therapeutic use in CNS disease and will undoubtedly yield further insight into the developmental plasticity and range of functions in this lineage, as well as providing additional investigative tools for ongoing study.

Therapeutic ablation of microglia in AD and ALS, where microglial activity has been shown to enhance disease severity, has been proposed in conjunction with engraftment of adoptively transferred myeloid cells (193). However, knowing

whether transferred myeloid cells will contribute to undesirable or unexpected adverse effects, due to their inability to mimic microglial behavior and perhaps fulfil microglial homeostatic roles, would be important to know. Other studies have attempted to generate microglia from human induced pluripotent stem cells in a defined media (194–197) to study human microglial behavior as well as for therapeutic prospects. Methods used to generate microglia are reviewed elsewhere (198). However, mRNA analysis showed that microglia from human induced pluripotent stem cells exhibited a phenotype similar to *in vitro* microglia rather than *ex vivo* microglia (150). More complex culturing conditions may be required to induce and maintain a microglial phenotype, including the use of organoids and co-culturing with glial cells (including astrocytes and oligodendrocytes). Understanding the specific gene-environment interactions that shape microglial phenotypes in different contexts will help inform ways to generate "microglia" as well as revealing what influences their phenotypic switch during disease. More recently, the development of human pluripotent stem cell (hPSC)-based microglia chimeric mouse brains, in which hPSC-derived cells are engrafted into neonatal mice, has evidently overcome the limitations of using cultured microglia to study these cells (199). Single-cell RNA sequencing data showed that these xenografted microglial cells resembled human microglia. Considering species-specific differences between microglia in humans and mice (150), this model provides a unique opportunity to study the role of human microglia in the intact brain.

Microglia, once considered a bystander of CNS physiology and pathology, are now in the spotlight of neuroimmune research. Single-cell protein and RNA sequencing technologies, *in-vivo* imaging and lineage-tracing techniques have substantially improved the delineation of myeloid populations in the CNS, as well as, our understanding of microglial physiology, ontogeny, and heterogeneity. This will likely elucidate their disease-related functions and inform targeted therapeutics.

AUTHOR CONTRIBUTIONS

AS, CW, and NK all contributed to the writing and conceptualization. CW was responsible for illustrating **Figure 1**. All authors contributed to the article and approved the submitted version.

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REVIEW
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The Gut-Brain Axis: How Microbiota and Host Inflammasome Influence Brain Physiology and Pathology

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The human microbiota has a fundamental role in host physiology and pathology. Gut microbial alteration, also known as dysbiosis, is a condition associated not only with gastrointestinal disorders but also with diseases affecting other distal organs. Recently it became evident that the intestinal bacteria can affect the central nervous system (CNS) physiology and inflammation. The nervous system and the gastrointestinal tract are communicating through a bidirectional network of signaling pathways called the gut-brain axis, which consists of multiple connections, including the vagus nerve, the immune system, and bacterial metabolites and products. During dysbiosis, these pathways are dysregulated and associated with altered permeability of the blood-brain barrier (BBB) and neuroinflammation. However, numerous mechanisms behind the impact of the gut microbiota in neuro-development and -pathogenesis remain poorly understood. There are several immune pathways involved in CNS homeostasis and inflammation. Among those, the inflammasome pathway has been linked to neuroinflammatory conditions such as multiple sclerosis, Alzheimer's and Parkinson's diseases, but also anxiety and depressive-like disorders. The inflammasome complex assembles upon cell activation due to exposure to microbes, danger signals, or stress and lead to the production of pro-inflammatory cytokines (interleukin-1β and interleukin-18) and to pyroptosis. Evidences suggest that there is a reciprocal influence of microbiota and inflammasome activation in the brain. However, how this influence is precisely working is yet to be discovered. Herein, we discuss the status of the knowledge and the open questions in the field focusing on the function of intestinal microbial metabolites or products on CNS cells during healthy and inflammatory conditions, such as multiple sclerosis, Alzheimer's and Parkinson's diseases, and also neuropsychiatric disorders. In particular, we focus on the innate inflammasome pathway as immune mechanism that can be involved in several of these conditions, upon exposure to certain microbes.

Keywords: microbiota, gut-brain axis, inflammasome, multiple sclerosis, Alzheimer's disease, Parkinson's disease, neuropsychiatric disorders

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INTRODUCTION

The mammalian intestinal microbiota comprises bacteria, viruses, fungi, yeasts, and bacteriophages. This community starts to develop at birth and continues for two–three years, in humans, until it reaches a stable composition (1). However, it continues to be influenced by different environmental and lifestyle factors throughout the lifetime. Therefore, the microbiota composition differs remarkably even between healthy individuals (2). Under healthy conditions, the microbiota influences numerous physiological processes within the host, such as protection against pathogens, nutrient digestion and absorption, development, and education of multiple host organs and the immune system (3–6).

In the last decade, different studies revealed strong associations between changes in the microbiota composition (a situation called "dysbiosis") and various host diseases (4, 5, 7). Interestingly, among those, there are also diseases affecting host organs in physical distance from the gut (8, 9), like the central nervous system (CNS) (7, 10–22). Moreover, a relevant contribution of gut microbiota is not only restricted to neuroinflammatory and psychiatric disorders (23–32) but also to brain development (13).

The CNS has long been considered an immune-privileged organ. The blood vessels that vascularize the brain are formed by endothelial cells firmly held together with tight junctions building the blood-brain barrier (BBB). The BBB allows to strictly regulate the movements of molecules, ions, and cells between the periphery and the brain (33). Importantly, the BBB protects the brain from pathogens and unwanted immune reactions that could damage the neurons and their connections (34). However, the idea that the CNS is an immune privileged organ has been reconsidered, as the functional immune cells can enter the CNS through the BBB, the choroid plexus, and the lymphatic vessels and have been described beyond neuropathological conditions (26, 35–47). Among the molecules that can pass the BBB are also bacterial products and metabolites shaping not only the CNS development and functions (6, 15) but also the genesis of certain diseases (33–42).

The communication between the CNS, the intestine, and the microbiota happens through the so called Gut-Brain Axis (GBA), a complex bidirectional communication network between the intestine and the CNS (10, 48). This axis involves different pathways such as the autonomic and enteric nervous system, the endocrine system, the hypothalamic-pituitary-adrenal axis (HPA), the immune system, and the microbiota and its metabolites (8, 31, 32). Several neurotransmitters (11, 49) and metabolites such as essential vitamins, secondary bile acids, amino acids, and short-chain fatty acids (SCFAs) (43, 46–51), modulate many immune system pathways (50–56) that in turn influence behavior, memory, learning, locomotion, and neurodegenerative disorders (45, 52–55). Among those pathways, researchers showed that the inflammasome plays a role in depressive- and anxiety-like behaviors, and locomotor activity (57). A potential role of dysbiosis has been suggested as cause of these mood and behavioral defects (55), however, the exact mechanism behind these phenomena still needs to be understood.

Despite growing evidence, a significant gap of knowledge still exists in understanding the exact mechanisms involved in the communication between gut and brain during health and disease. In this review, we provide an overview of the current state of research about the effect of microbiota on the GBA in homeostasis and disease states, with a particular interest in the different bacterial metabolites involved. We further discuss the potential contribution of inflammasomes on the GBA, highlighting the critical open questions that remain in the field.

Abbreviations: CNS, Central nervous system; BBB, Blood-brain barrier; GBA, Gut-Brain Axis; HPA, Hypothalamic-pituitary-adrenal axis; SCFAs, Short-chain fatty acids; SPF, Specific pathogen-free; GF, Germ-free; 5-HT, 5-hydroxytryptophan; PSD-95, Postsynaptic density protein 95; GI, Gastro-intestinal; Ahr, Aryl hydrocarbon receptor; SFB, *Segmented Filamentous Bacterium*; ASF, Altered Schaedler Flora; MS, Multiple sclerosis; AD, Alzheimer's disease; PD, Parkinson's disease; NPS, Neuropsychiatric disorders; FMT, Fecal microbiota transplantations; EAE, Experimental autoimmune encephalomyelitis; PSA, Polysaccharide A; Treg, T regulatory; Th, T helper; Trp, Tryptophan; I3S, Indoxyl-3-sulfate; IPA, Indole-3-propionic acid; IAld, Indole-3- aldehyde; Aβ, Beta-amyloid protein; APP, Amyloid precursor protein; FAD, Familial Alzheimer's Disease; ASO, Overexpressing α-synuclein under Thy1 promoter; α-syn, α-synuclein; MDD, Major depressive disorder; ASD, Autism spectrum disorder; OTUs, Operational taxonomy units; 5-AV, 5-aminovaleric acid; MIA, Maternal immune activation; 4EPS, 4-ethylphenylsulfate; PRRs, Pattern-recognition receptors; NLRs, NOD-like receptors; TLR, Toll-like receptors; ASC, Apoptosis-associated speck-like protein; PAMPS/DAMPS, Pathogen- or danger-associated molecular patterns; SlrP, *Salmonella* leucine-rich repeat protein; PBMCs, Peripheral blood mononuclear cells; CSF, Cerebrospinal fluid; PTX, Pertussis toxin; HSV-1, *Herpes simplex virus type 1*; hiNSC, Human-induced neural stem cell; PINK1, Mitochondrial serine/threonine protein kinase; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; SSD, Schizophrenia spectrum disorders; OCD, Obsessive-compulsive disorder; NSSID, Non-suicidal self-injury disorder; BMI, Body mass index; BHB, Beta-hydroxybutyrate; TCR, T cell-receptor; MOG, Myelin-oligodendrocyte glycoprotein.

HOST-MICROBIAL MUTUALISM IN THE GUT-BRAIN AXIS: ROLE OF BACTERIAL MOLECULES AND METABOLITES IN DEVELOPMENT AND HEALTH

The microbiota is a community of commensal and symbiotic microorganisms that reach a density of more than 10^{12} cells/g of content in the human large intestine (16). 500 to 1,000 different bacterial species populate the mammalian gut, belonging to the four dominant bacterial phyla Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria. A well-balanced beneficial interaction between the host and its microbiota is an essential requirement for intestinal health and the body as a whole. Under healthy conditions, the mucosal microbiota plays a vital role in food digestion, vitamin synthesis, angiogenesis, epithelial cell maturation, development, education of the host immune system, and protection against pathogens (58–66). Notably, the microbiota orchestrates the local immune system in the intestine (67), and shapes immune and non-immune cells located in distal sites and acting systemically (68, 69).

Colonization of the intestine with a unique microbial community starts at birth through the exposure of the infant

to the microflora of the vaginal tract and the mother's skin. The microbiota develops and gets stable by the age of 2 to 3 years in humans (65) and within 3 to 4 weeks of life in mice (70). This early life window corresponds to a period in which several organs of the body go through critical phases of development and growth (71). During this period, the infant's immune system develops and the host microbiota matures and stabilizes. The microbiota first gets into contact with the immune system on the mucosal sites, shaping the immune tolerance to commensal microbes and establishing mucosal integrity at the same time. Together with these events, also distal organs get affected. The brain, in particular, undergoes dramatic changes within the early life period. Within the first three months of life in humans, its size increases more than 50% from the time of birth, reaching 90% of the size of the adult organ within the first five years of life (72). In this period, neuronal development takes place (73) and it is supported and shaped by maternal microbiota (74–79).

The influence of the intestinal microbiota in neurodevelopment was known since early 2000s. Early experiments using germ-free (GF) or specific pathogen-free (SPF) mice treated with antibiotics, to reduce the microbial diversity within the intestine, showed that several neurological problems occur in mice with reduced or lack of proper mature gut microbiota (7, 10, 14, 15, 34, 80, 81). In details, compared to colonized mice, GF mice showed exaggerated hypothalamic–pituitary–adrenal (HPA) restrain stress reaction (10), impared social behaviors (12, 15, 82), reduced anxiety-like behavior (7, 81–83) and increased motor and rearing activity (80, 84). Consistently, certain altered brain developments and behaviors observed in GF mice could be resolved/improved when new born animals were reconstituted with a diverse and intact flora (73, 82, 83). Antibiotic treatment results in reduced expression of the tight-junction forming proteins, occludin, and claudin-5, in the brain, increased BBB permeability, reduced anxiety-like behaviors, and elevated exploratory behavior and home-cage activity (35). The altered behavioral phenotype was associated with dysregulation of genes and metabolites known to be involved in motor control and anxiety-like behavior pathways, like adrenaline, dopamine, 5-hydroxytryptophan (5-HT), postsynaptic density protein 95 (PSD-95), and synaptophysin (80).

Lately, it is becoming more evident that microbes can produce neuroactive molecules that directly contribute to the communication between the gut and the brain (**Figure 1**). Neurotransmitters, such as acetylcholine, GABA, and serotonin, produced by bacteria belonging to *Lactobacillus, Bifidobacteria*, *Enterococcus*, and *Streptococcus* species, can influence brain cell physiology directly and indirectly (11, 85, 86). Strikingly, 90% of serotonin required for mood, behavior, sleep, and several other functions within the CNS and gastrointestinal (GI) tract is produced in the gut (87). Binding of serotonin to 5-HT receptors on microglia induces the release of cytokine-carrying exosomes, providing another mechanism for gut-induced modulation of neuroinflammation (88). Another microbial metabolite that influences microglia activity is tryptophan, a serotonin precursor (89). Bacterial metabolites derived from dietary tryptophan could control the CNS inflammation through an aryl hydrocarbon receptor (Ahr)-mediated mechanism acting on microglial activation and the transcriptional program of astrocytes (89). The importance of tryptophan metabolism in maintaining CNS homeostasis was already known a few years earlier, since male GF animals have significantly higher levels of 5-hydroxytryptamine and 5-hydroxyindoleacetic acid in the hippocampus and the serum, compared with conventionally colonized control animals (7). These findings suggest that the systemic circulation could be the route through which the microbiota influences CNS serotonergic neurotransmission. Interestingly, colonizing GF animals post-weaning was sufficient to restore the levels of tryptophan in the periphery and to reduce anxiety in GF animals, but was insufficient to reverse the CNS neurochemical consequences present in adult GF animals (7). This approach highlighted once more the importance of an intact and diverse microbiota from birth on. More recently, it has also been reported that metabolism of tryptophan by activated microglia produces the neurotoxin quinolinic acid, an *N*-methyl-D-aspartate agonist, implicated in several neurological conditions, including Huntington's disease and depression (90). Recolonizing GF mice with particular bacteria belonging to the Clostridia family, such as *Clostridium tyrobutyricum*, known to colonize the intestinal mucus layer, regulates immune and gut barrier homeostasis through the production of anti-inflammatory metabolites (e.g. butyrate), induces elevation of occludin and claudin-5 levels in brains of GF mice and restores their BBB integrity to the level of SPF mice (91). Furthermore, probiotic supplementations, as *Lactobacillus rhamnosus* (JB-1), in already colonized mice, reduced anxiety- and depression-like behavior in steady-state conditions (92, 93). In 2019, Artis D.'s group showed that SPF mice treated with a cocktail of broad-spectrum antibiotics, GF mice, GF mice recolonized after weaning age with a simple microbiota or a complex microbiota, have defects in fear extinction learning, compared to SPF mice or GF mice colonized with SPF flora at the time of birth (29). Fear extinction learning is a reaction that happens after experiencing an environmental danger and has been implicated in multiple neuropsychiatric disorders, including anxiety disorders like post-traumatic stress disorder (29). The reasons for this altered behavioral response in the absence of a diverse and intact microbiota were reconducted to alterations in pathways involved in synapse formation and calcium signaling at the level of mainly neuronal and microglial cells (29). The researchers showed that the microbiota-mediated changes in synapse formation and fear extinction behavior were not the results of the hypothalamic-pituitary-adrenal axis but of the reduced level of potential neuroactive metabolites (phenyl sulfate, pyrocatechol sulfate, 3-(3-sulfooxyphenyl)propanoic acid, and indoxyl sulfate) in the cerebrospinal fluid, serum and in fecal samples of GF mice compared to SPF mice (29). However, the types of cells (host or bacterial) producing these metabolites are still undiscovered.

From an immunological and metabolomic point of view, GF, SPF mice treated with antibiotics, or gnotobiotic mice with limited microbiome diversity (colonized with ASF for example) showed impaired microglia maturation and immune response

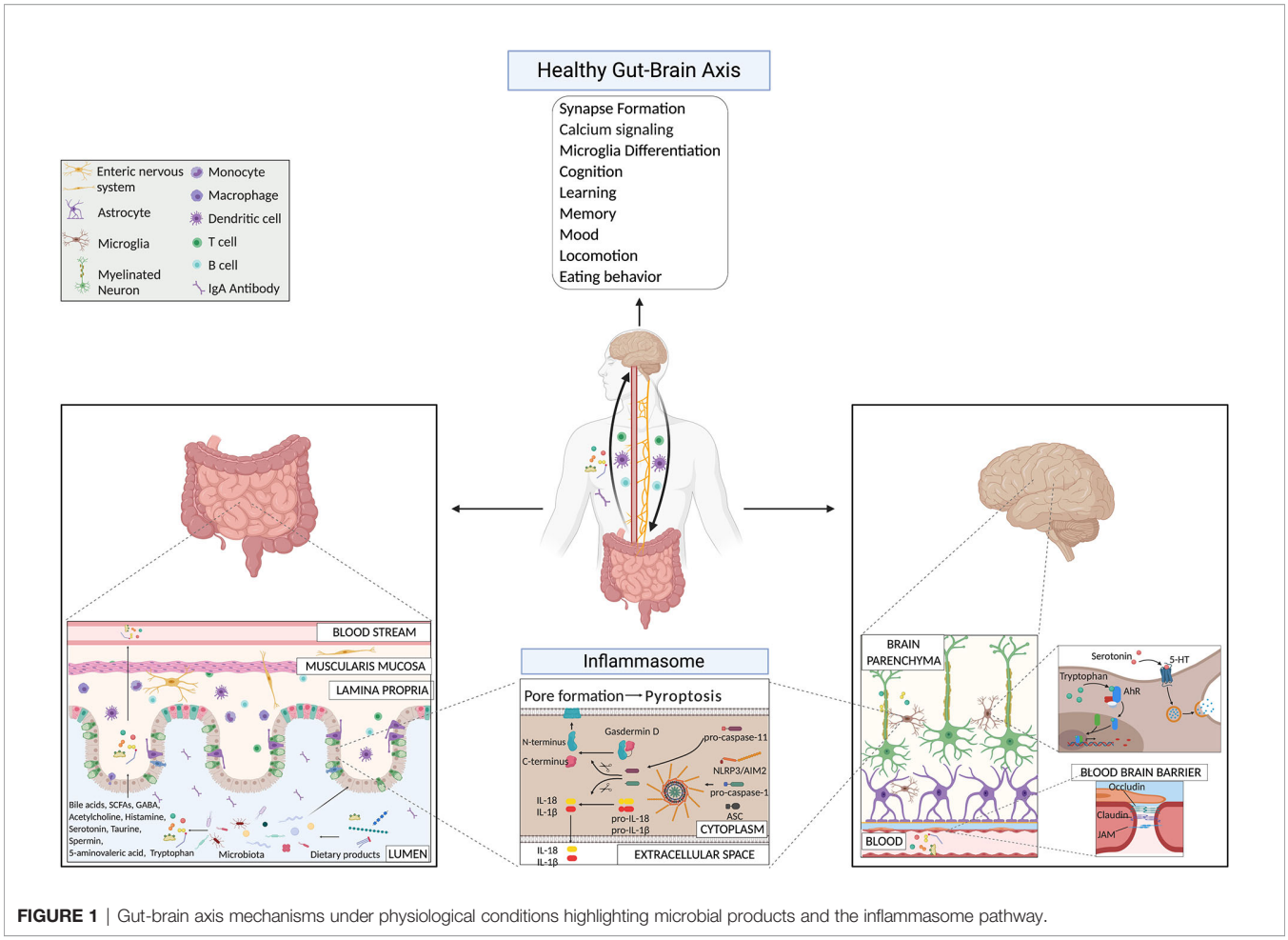

FIGURE 1 | Gut-brain axis mechanisms under physiological conditions highlighting microbial products and the inflammasome pathway.

upon bacterial stimuli, compared to SPF mice (94). Moreover, the treatment of mice with *E. coli*, isolated from colitic mice, caused colitis and brain memory impairment (95, 96). In contrast, the treatment with *L. johnsonii* restored a healthy gut microbiota composition and attenuated both colitis and *E. coli*-induced memory impairment (95, 96). In addition, bacterial fermentation of indigestible dietary fibers produces among the SCFAs, butyrate, propionate, and acetate in the colon (97). SCFAs maintain gut health by promoting intestinal barrier integrity, mucus production, and supporting a tolerogenic response over inflammation (49, 50, 98, 99). However, their activity is not restricted only to the intestine. A small fraction reaches the systemic circulation and can cross the tightly regulated BBB using their own transporters located on brain vascular epithelial cells (100, 101). SCFAs are, in fact, detectable in low amounts in the human brain under physiological conditions (102). Additionally, they also affect the BBB itself; the colonization of adult GF mice with a complex microbiota or only with SCFAs-producing bacterial strains restores the integrity of the BBB (91). Remarkably, treating GF mice with the oral application of a mixture of the three major SCFAs acetate, propionate, and butyrate, was also sufficient to restore the normal maturation process of the microglia (94). Moreover, SCFAs can modulate neurotransmitters, like glutamate, glutamine, GABA, and neurotrophic factors (103). Propionate and butyrate can influence the cell signaling system *via* modification of the intracellular potassium levels (104), and they regulate the expression levels of tryptophan 5-hydroxylase 1, involved in the synthesis of serotonin, and tyrosine hydroxylase, which is involved in the biosynthesis of dopamine, adrenaline, and noradrenaline (105).

Several other immune pathways have been shown to affect behavior, memory, learning, and locomotion (41, 57, 106–108). Among those, we will discuss the role of the inflammasome pathway in the GBA in more details later on.

NEUROLOGICAL DISEASES: MICROBIAL EFFECT ON THE HOST IMMUNE AND NERVOUS SYSTEM

Several poorly understood environmental factors, including dietary and habit factors, have been linked to susceptibility to neurological disorders and alterations in the gut microbiota (30, 109, 110). The microbiota composition differs significantly between healthy controls and patients affected by neurodegenerative disorders (such as multiple sclerosis (MS), Alzheimer's (AD) and

Parkinson's (PD) diseases) (20, 22, 23, 32, 111–115), and neuropsychiatric disorders (NPS) (30), like major depressive and mood disorders. Of extreme relevance, the altered microbiota of patients could transfer the disease from a human host to a mouse host (113, 116–120). Here, we present the mechanisms driven by the bacteria that induce different neurological diseases (**Figure 2**). We are at the initial phases of this discovery path, and for the majority of the pathological conditions, we still do not know if the dysbiosis is the cause or rather the consequence of it. Here, we focus our attention on the works that suggested mechanisms of action by bacteria in the etiology of certain CNS disorders.

Multiple Sclerosis

Multiple sclerosis (MS) is an autoimmune and neurodegenerative disease affecting more than two million people worldwide. This condition is characterized by neuroinflammation, infiltration of lymphocytes into the CNS, demyelination, and axonal loss. Clinical signs associated with MS include ataxia, loss of coordination, hyperreflexia, spasticity, visual and sensory impairment, fatigue and cognitive difficulties. Most of the patients have a relapsing-remitting form of the disease, characterized by a progressive relapse of the symptoms with increased severe neurological deterioration over time (121). The majority of the patients develop lesions in the brain or in both brain and spinal cord, although few develop lesions in the spinal cord only (121). MS is the cause of death in more than 50% of the affected patients (122). Factors involved in the pathogenesis are both environmental and genetic (123–131). Among the environmental factors, microbes (and their secreted products or toxins) play a critical role in the pathogenesis of MS (132, 133). Several relevant pioneer studies showed that external microbial infections and intestinal commensal bacteria can be involved in disease development.

First of all, the microbiota composition of MS patients is different from the one of healthy individuals (104). Interestingly, even MS patients with active disease have an altered microbiota compared to patients in the remission phase, which in turn have a microbiota more similar to healthy controls (25, 134–138). Bacteria belonging to the Clostridia family contribute to the suppression of pathological autoimmunity (134, 139–143). Higher abundance of Firmicutes and the absence of Fusobacteria were associated with a shorter time to relapse in pediatric MS patients (144). Additionally, MS patients that were treated with the antibiotic minocycline, a broad-spectrum tetracycline, have reduced rate of relapses and amelioration of several immunological parameters (such as IL-12p40, metalloproteinase-9, and soluble vascular cell adhesion

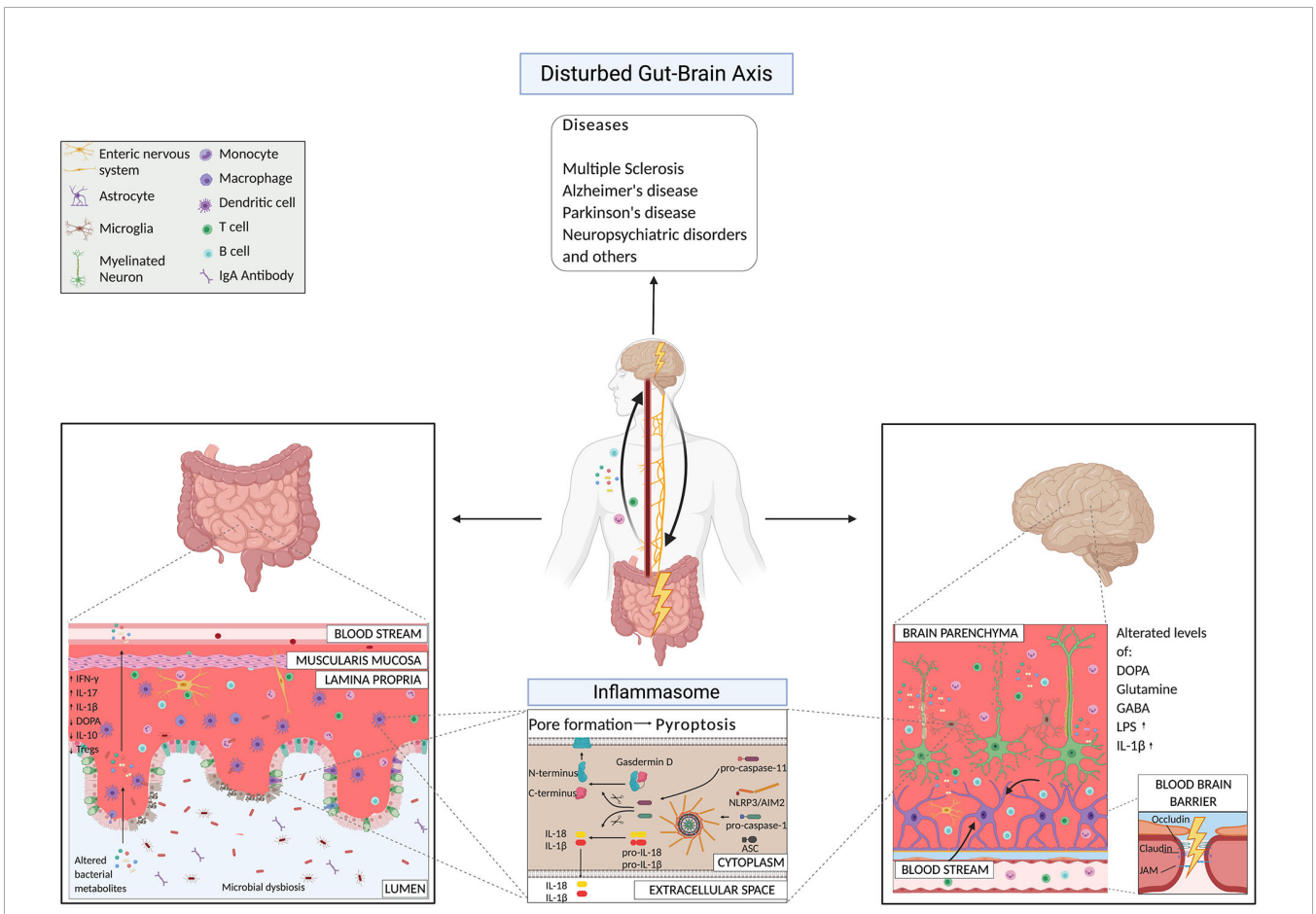

FIGURE 2 | Gut-brain axis mechanisms under pathological conditions highlighting microbial products and the inflammasome pathway.

molecule-1) (145, 146). However, the minocycline effect on the patient microbiota composition was not addressed. In an additional relevant study, three MS patients underwent multiple fecal microbiota transplantations (FMT) to treat severe constipation. This treatment reverted the intestinal illness and improved the MS symptoms (147), proving the existence of a gut-brain connection. Moreover, probiotics supplementations have a therapeutic potential to ameliorate MS, improving the disability status, the mental health, and some inflammatory and metabolic parameters, compared to the placebo group (148, 149). The seminal study by Berer K. and colleagues highlighted the strong immunological *in vivo* impact of the microbiota on MS pathogenesis (112). They recruited a cohort of monozygotic twin couples in which one individual was affected by MS and the other twin was healthy (112). They reported that MS-twins had a higher abundance of taxa like *Akkermansia* in their feces (112). However, the remarkable finding was that transplanting the MS-twins' intestinal microbes into GF animals, genetically susceptible to developing experimental autoimmune encephalomyelitis (EAE) was enough to promote the disease *in vivo* with a significantly higher incidence than by transplanting the healthy twins' microbes (112). Interestingly, the immune cells of murine recipients of MS-derived samples produced less IL-10 than cells from mice colonized with the microbiota derived from the healthy twins (112). IL-10 is one of the master regulatory cytokines, and its neutralization in mice colonized with healthy fecal samples increased disease incidence (112). This significant finding highlighted the potential of the human microbiota to induce specific immune system alterations that might be the cause or the effect of the development of MS. However, the exact mechanisms that suppress the production of regulatory cytokines in the hosts receiving certain microbes from MS patients are still unclear. Several investigations in animals started to elucidate these mechanisms. Kasper L.'s group showed that oral but not systemic treatment with a classical pool of broad-spectrum antibiotics (ampicillin, vancomycin, neomycin, and metronidazole) ameliorated the development of acute EAE, in two mouse models (on C57BL/6 and SJL backgrounds) (18). The better disease outcome upon oral antibiotic treatment was associated with a reduction of pro-inflammatory cytokines and an increase in regulatory cytokines like IL-10 (18). This effect suggested that the intestinal microbiota was responsible for the disease severity and the modulation of the adaptive immune response during disease development (18).

One year later, the same laboratory showed how a single bacterium could affect EAE pathogenesis. They used a similar approach in mice by depleting the intestinal bacteria *via* oral antibiotic treatment followed by the reconstitution only with *B. fragilis*, either wild-type (WT) or deficient for the production of the zwitterionic capsular polysaccharide A (ΔPSA) (116). Mice treated with antibiotics alone or recolonized with the PSA-wild-type strain of *B. fragilis* were protected against disease, whereas the mice recolonized with ΔPSA *B. fragilis* and as well the vehicle-treated control group developed the disease (116). The authors also understood that *B. fragilis* induced EAE protection through the generation of IL-10–producing T regulatory (Treg) cells in a PSA-dependent manner (20). They then concluded that oral treatment with PSA could cause protection from EAE in mice, when used as both prophylactic and therapeutic approach, *via* recruitment of $CD103^+$ $CD11c^{high}$ antigen-presenting cells and priming of IL-10–producing Treg cells in the cervical lymph-nodes (150). Thereafter, Lee Y. and colleagues showed for the first time that GF mice were almost completely protected from EAE compared to conventionally colonized mice (117). Again, the protection was associated with a decrease of pro-inflammatory cytokine levels, such as IFN-γ and IL-17A, and an increase in Treg cells in the peripheral organs, the gut and the spinal cord (117). Strikingly, GF mice colonized with only a single bacterium, the SFB, developed EAE, and a T helper (Th)-17 pathogenic immune response in the gut and in the CNS (117).

In the recent milestone study from the groups of Gommerman J. and Baranzini SE., IL-10- and IgA-producing plasma cells have been suggested to have a role in ameliorating EAE in mice and to correlate with relapse in MS patients (151). Microbial specific IgA-secreting cells were shown to be present in both the bone marrow and, in particular, in the brain of EAE-affected mice but not in healthy animals. Remarkably, MS patients during active relapse had less IgA specific for intestinal bacteria compared to patients in remission, suggesting the capacity of IgA-producing cells to migrate from the gut to the central nervous system during disease relapses, as in the mouse system (151). The authors also ruled out one of the potential mechanisms mediated by IgA-secreting plasma cells to suppress EAE. Briefly, commensal-reactive IgA-producing cells expressed IL-10, and the production of IL-10 (partially together with iNOS, but not IgA itself) was essential to ameliorate EAE (151). Lately, in the classical acute EAE model, ampicillin administration alone ameliorated the EAE development (152) with a reduction of proliferating $CD4^+$ autoreactive T cells. Miyauchi E. and colleagues showed that the *in vivo* treatment with ampicillin induced the complete depletion of *Allobaculum* bacteria from the small bowel. Monocolonization with this bacterium induced the generation of Th17 cells in the small intestinal lamina propria and systemically, and increased severity of EAE, compared to GF mice (152). However, the disease development was less severe than under conventional hygiene conditions. The authors focused then their attention on bacteria homing to the small intestine that could favor the generation and proliferation of autoreactive T cells *via* presentation of cross-reactive antigens. Mice bearing a T cell-receptor (TCR) specific for myelin-oligodendrocyte glycoprotein (MOG) (2D2 mice) showed higher number of $CD4^+$ T cells in the small intestinal lamina propria compared to their wild-type counterpart (152). Some candidate mimicry peptides, like UvrABC system protein A (UvrA), were expressed by *L. reuteri*, and aminopeptidase by strains of *Allobaculum* (152). Interestingly, *L. reuteri* monocolonization did not affect the severity of EAE. However, the bi-colonization with *Allobaculum* and *L. reuteri* together activated MOG-specific T cells toward a pathogenic Th17 phenotype and worsened EAE, with demyelination and cell

infiltration in the spinal cord (152). This study highlighted the important synergistic potential of several intestinal bacteria, along the entire intestinal tract and perhaps residing even in close contact with the mucus layer, in mediating the disease development through molecular mimicry.

Altogether, these breakthrough murine studies prove that certain intestinal bacterial species profoundly affect EAE pathogenesis, altering the balance between pro- and anti-inflammatory immune responses *via* a direct effect of some bacterial products. Treating mice that spontaneously develop EAE with a cocktail of broad spectrum antibiotics resulted in dramatically different outcomes, depending on whether the antibiotics were given before or after the onset of the autoimmune disease. Prophylactic antibiotic treatment (at 1–3 weeks before the clinical onset of the disease, starting from 2–4 weeks of age) led to a significant reduction of susceptibility to spontaneous EAE, accompanied by altered intestinal microbial composition and a decrease in Th-17 cell development (153). In contrast, antibiotic treatment after the onset of the first signs of spontaneous EAE did not affect the ongoing disease and CNS inflammation (153). The supplementation of a cocktail of five probiotics (IRT5) before or after the EAE onset in rodents is associated with the delay of the disease onset or suppression of the disease progression, respectively (154). The amelioration of the disease upon probiotic treatment was associated with a higher abundance of Treg cells, higher production of IL-10 by $CD4^+$ T cells, B cells, and $CD11c^+$ cells, together with the suppression of the pro-inflammatory Th-1/Th-17 response (154). These results suggest that microbiota modulation in early life stages or prophylactic treatments in subjects that are genetically prone to develop certain autoimmune diseases, such as MS, could be efficient approaches to ameliorate the disease susceptibility and/or to delay the onset of the disease. Notably, only certain bacteria with specific metabolic or structural characteristics appear effective in preventing or postponing the onset of certain conditions. Such as, oral administration of SCFAs ameliorate EAE development and disease severity (155, 156). Mechanistically, SCFAs favor the enhancement of acetyl-CoA metabolism, histone acetylation, preservation of spinal cord lipid content, suppression of demyelination, oligodendrocyte maturation and differentiation (157, 158). As also mentioned earlier, in the last years, Quintana F.'s group provided an exceptional contribution to the field, revealing the role of dietary tryptophan (Trp) metabolism in MS pathogenesis (118). The gut microbiota metabolizes dietary tryptophan into AhR agonists (119). Firstly, they showed that treating mice with antibiotics or feeding them with a Trp-deficient diet during the recovery phase of the EAE model worsened the EAE scores (118). However, the disease was ameliorated by supplementation of Trp metabolites (like indole, indoxyl-3-sulfate (I3S), indole-3-propionic acid (IPA) and indole-3-aldehyde (IAld)) or feeding with Trp-enriched diet (118). They also found that in patients affected by MS, the circulating levels of AhR agonists are decreased. In conclusion, they suggested a mode of action of Ahr agonists, *via* a direct effect on astrocytes to limit CNS inflammation during EAE (118). A couple of years later, the same group discovered that the microglia has a crucial role in this mechanism of protection from EAE *via* the Trp-AhR pathway (89). Briefly, Trp or I3S treatment ameliorated EAE scores in control mice but not in mice lacking AhR expression on the microglia fed with Trp-deficient diet (89). This treatment was initiated 14 days after EAE induction and ameliorated the disease *via* AhR engagement on astrocytes and microglia (89). Moreover, using primary human microglia, they showed that the *in vitro* stimulation with I3S activated the AhR signaling pathway leading to the suppression of pro-inflammatory pathways (via TNF-α, IL-6, IL-12A, NOS2, VEGF-β expression), and the promotion of anti-inflammatory responses (such as IL-10 and TGF-α expression) (89). Finally, they detected AhR, TGF-α, and VEGF-β expression on myeloid $CD14^+$ cells in the demyelinated active and chronic MS lesions of patients (89). These findings suggest that metabolites derived from the digestion of tryptophan by the gut flora activate AhR signaling in astrocytes and microglia and induce immune protection mechanisms in the host that are important to suppress CNS inflammation, in both animal models and perhaps also in human patients. Altogether, these works suggest the efficacy of different interventions aimed to modify the microbiota composition as prophylactic or therapeutic approaches. Therefore, the need of studies that help to understand the mechanism of action of different microbial methods in different patients, bearing a diverse microbiota, becomes a priority for future research in the field.

Alzheimer's Disease

Alzheimer's disease (AD) is the most common cause of progressive dementia that affects nearly 50 million people worldwide. Symptoms affecting memory and thinking become, with time, critically severe compromising even the simplest daily life tasks. Until today, neither a therapeutic nor a prophylactic strategy exists against this devastating neurodegenerative disorder (159). AD is caused by the formation of aggregates of polymerized forms of β-amyloid precursor protein (Aβ) in soluble multimeric and/or insoluble amyloid deposits in the brain, that trigger a cascade of pathological events leading to neurofibrillary tangles, aggregates of hyperphosphorylated tau proteins, formation of neurofibrillary lesions, and ultimately dementia (159). Several microbial factors have been linked to the AD pathogenesis (140, 141), and a few studies suggested alteration in the commensal microbiota and pathogenic infections as potential causes of AD (142).

Stool microbial profile of AD patients display decreased numbers of Firmicutes and Actinobacteria, and increased Bacteroidetes compared to controls. Within the Firmicutes, the families *Ruminococcaceae*, *Turicibacteraceae*, and *Clostridiaceae* were all less abundant in AD patient (160).

APP/PS1 double transgenic mice, which express special neurons in the CNS with a chimeric mouse/human amyloid precursor protein (APP) and a mutant human presenilin 1 (PS1), show a remarkable shift in the gut microbiota composition compared to healthy wild-type mice (161). Fewer Firmicutes (*Akkermansia* and *Rikenellaceaea*) but more Bacteroidetes (S24-7) were identified in conventionally-raised APP/PS1 mice compared to wild-type littermate controls (161). Importantly, GF-APP/PS1 transgenic mice have reduced Aβ levels in the brain and blood and reduced amyloid load compared to

conventionally-raised APP/PS1 mice (161). Fecal transplantation from conventionally-raised APP/PS1 mice into GF APP/PS1 hosts dramatically increased cerebral Aβ pathology in the hosts (161). Altogether, these results strongly supported a microbiota involvement in the development of AD in AD-susceptible animal models (161). Additionally, 5xFAD mice (over-expressing in the brain the mutant human APP carrying Swedish, Florida, and London Familial Alzheimer's Disease (FAD) mutations along with human PS1 harboring two FAD mutations) recapitulate significant AD and fecal microbial composition features that evolve with age (162, 163). At the age of two–three months, when Aβ plaque deposition in the cortex and hippocampus starts, Bacteroides, Firmicutes, and Verrucomicrobia were the most abundant bacterial phyla (163). At the age of seven months, when the signs of synaptic degeneration appear, Firmicutes became the predominant phylum, with a marked decrease in abundance of Bacteroidetes and Verrucomicrobia. These variations over time were absent in wild-type mice, which had a much more stable microbiota throughout the lifespan (163). These changes in microbiota composition were associated with neuroinflammatory markers. In fact, at 2 to 3 months of age, both pro-inflammatory and anti-inflammatory microglia expanded, while in the following months, only the first subset continued to grow, along with the recruitment of Th-1 $CD4^+$ proinflammatory cells, reaching a peak at seven-nine months. On the other hand, the anti-inflammatory microglia declined from three to five months and maintained at low levels after that. Importantly, this was the first time that changes in the microbiota composition have been monitored during a neurological disease development suggesting that the bacterial changes could be happening together or before the immunological and neurological changes. The neuroinflammatory modifications were, in fact, strikingly dependent on the microbiota ones, and the depletion of intestinal microbes *via* antibiotic treatment ameliorated the recruitment and priming of anti-inflammatory microglia and Th-1 cells (163). Co-housing or FMT experiments showed that neuroinflammation and cognitive impairments could be transferred from 5xFAD mice to wild-type counterparts (163). The usage of a sodium oligomannate, with known cognition improvement effect in humans, suppressed neuroinflammation, Aβ plaque deposition, and cognition impairment (164). However, from the microbiota point of view, the most striking result of this therapeutic approach comes from the FMT experiments from oligomannate-treated 5xFAD mice into WT hosts that were pre-treated with Aβ aggregate injections to induce AD development (163). The feces isolated from oligomannate-treated 5xFAD mice transferred protection from neuroinflammatory events in recipient animals (163). Metabolomic analyses on the feces of these animals revealed significant changes in amino acid–related metabolism, in particular for the phenylalanine- and isoleucine-related pathways. Phenylalanine and isoleucine can be uptaken by adaptive immune cells, like Th-1 cells (163). In addition, the intestinal microbiota diversity was altered and the levels of SCFAs were reduced in AD mice compared to wild-type control mice. Therefore, modifications of the intestinal flora impact several metabolic pathways in AD mouse models, that could be leading to cognitive defects, amyloid deposition, and intestinal abnormalities (165). Similarly, a recent work showed that $ADLP^{APT}$ mice present community level-alterations in the microbiota compared to wild-type animals (166). The $ADLP^{APT}$ mice carry six human mutations affecting amyloid precursor protein, presenilin-1, and tau protein, and develop an AD-like pathology with amyloid and neurofibrillary tangles (167). Upon fecal microbial transplantations from WT animals into $ADLP^{APT}$ mice, formation of amyloid β plaques and neurofibrillary tangles, glial reactivity and cognitive impairment were ameliorated in the recipients mice (166). Together, these findings highlight the role of gut microbes in the promotion of neuroinflammation in AD progression through alteration in metabolic and immunological pathways.

Probiotics supplementation has been taken into consideration also for AD (168, 169). The human isolate *Bifidobacterium longum* (NK46) was orally administered in 5xFAD mice and induced anti-inflammatory effects (decrease in lipopolysaccharide (LPS) levels, NF-κB activation, and TNF-α expression), changes in the intestinal microbiota composition of the recipients (increase in Bacteroides and reduction in Firmicutes and Proteobacteria phyla), and suppression of Aβ accumulation in the hippocampus (170).

To conclude, an intriguing and relevant role of the oral pathogen *Porphyromonas gingivalis*, the causing agent of chronic periodontitis, has been elucidated in the etiology of AD (120). We think that the mechanisms adopted by this bacterium could be relevant to study as also some commensals could have a similar mode of action. Dominy SS. and colleagues identified *Porphyromonas gingivalis* and the gingipains, the toxic proteases produced by this bacterium, in the brain of AD patients but not in the brain of control patients with no history of any neurological abnormality or condition (120). Gingipains co-localized with neurons and astrocytes and also tau tangles and intraneuronal Aβ in the tissue of AD patients (120). *P. gingivalis* 16S rRNA was detected in both the cerebrospinal fluid and in the cerebral cortex of AD patients (120). The oral *P. gingivalis* infection in mice that are not genetically susceptible to develop AD resulted in the detection of *P. gingivalis* DNA in the brain and remarkably increased production of Aβ deposits (120). On the other hand, mice that received gingipains-deficient *P. gingivalis* or synthetic gingipains inhibitors had significantly less *P. gingivalis* DNA detectable in the brain, less Aβ production, reduced neuroinflammation, and increased number of healthy neurons in the hippocampus (120). For the first time, some bacterial molecules, as gingipains, have been identified as neurotoxic and having a critical role in the generation of an Aβ response *in vivo* (120). These findings also suggest gingipain inhibitors as valuable instrument for treating *P. gingivalis* brain colonization and potentially even the neurodegeneration in AD (120). This breakthrough study also suggested that some bacteria could impact the physiology of the brain or other tissues by reaching them alive, if specific barriers are leaking or certain conditions will happen, or dead, or by releasing soluble factors. More studies are needed to address the exact mechanism through which this is happening and under which circumstances. It could be relevant to study if mechanisms adopted by this bacterium in the oral cavity are similar to the ones used by commensals in other sites.

Parkinson's Disease

Parkinson's disease (PD) is the second most common neurodegenerative disorder, affecting ten million people worldwide (171). It is a progressive nervous system disorder that affects movement. Symptoms start gradually, from a very mild and hardly recognized tremor in one hand, to reach stiffness or slowing of movements, difficulties in walking, accompanied by cognitive and behavior defects (172). PD affects predominantly dopaminergic neurons in the substantia nigra of the brain, leading to a loss in control and coordination of the movements (172). Both genetic and environmental factors have been linked to the etiology of the disease. Genetically, toxic protofibrils formation, consisting of soluble oligomers of α-synuclein, a presynaptic protein, has been involved in the disruption of synaptic functions and in neuronal death. Targeting α-synuclein (α-syn) has been taken into consideration to develop therapeutic strategies in PD and other synucleinopathies (173). However, still nowadays, there is no cure for this neurodegenerative disorder.

As with most of the diseases, PD patients have a different microbiota composition compared to healthy controls or patients affected by other neurological disorders (174–176). Remarkably, PD patients harbored an intestinal flora depleted in SCFAs (mainly butyrate)-producing bacteria, like taxa from Lachnospiraceae family (175, 177–179), and *Faecalibacterium prausnitzii* (175, 180), those with known anti-inflammatory properties. Those butyrate-producing bacteria are also associated with a decrease in the dopamine metabolite and lower quality of life and signs of depression (22). This finding suggests that a PD-typical microbiota composition could be associated with less dopamine production and PD. Another important characteristic of the disease is a disrupted intestinal barrier, which leads to the systemic dissemination of microbial products like LPS and an increase in expression of intestinal pro-inflammatory genes (181). An essential role of the microbiota in PD pathogenesis has been shown in animal models. GF mice colonized with microbiota from PD patients display physical impairments compared to GF mice colonized with microbiota from healthy human donors (111). Importantly, ASO (overexpressing a-synuclein under Thy1 promoter) mouse model of PD develops progressive deficits in motor function as well as in gut motility (182, 183). ASO GF mice show reduced signs of PD and diminished α-synuclein aggregates in the frontal cortex, but not in the cerebellum, compared to their SPF counterparts (103). Antibiotic treatment of SPF ASO mice induced very mild α-syn-dependent motor dysfunction, like in mice born under GF conditions (103). Moreover, colonization of ASO GF animals with SPF flora at five-six weeks of age recapitulated the significant motor dysfunction observed in ASO SPF mice (103). Importantly, the microglia phenotype and also the gastrointestinal function (measured as fecal output) were significantly improved in antibiotic-treated animals but diminished in GF mice recolonized with SPF flora (103). Remarkably, ASO-GF mice treated with SCFAs mixture showed again mature microglia, formation of α-syn-aggregates, GI deficits, and significantly impaired performance in several motor tasks, as ASO-SPF mice (103). Host exposure to dead bacteria was not sufficient to induce the pathogenesis in the ASO-GF mice (103). Moreover, the simple treatment with minocycline was adequate to reduce inflammation, α-syn-aggregates, and improve motor function (103). Altogether, this work suggests that only live bacteria, producing active metabolites, such as SCFAs, could promote inflammation and development of the disease in the ASO model. These findings highlight the fact that the same bacterial metabolite could have a protective effect on specific disease models and a devastating impact on different ones. Therefore, it is vital to understand how these substances work in different conditions. Transferring PD patient-derived feces to GF mice was of high relevance. The differences in the microbiome profile, in SCFAs amount, and motor dysfunctions (increase in propionate and butyrate) were transferred and maintained *in vivo* too (111). In a different PD animal model, the pesticide rotenone-induced mouse model, researchers reported significant changes in the composition of caecum mucosal-associated and luminal microbiota, with a decrease in abundance of *Bifidobacterium* genus. These differences were associated with alterations in the metabolic pathways expressed by the commensal bacteria (184). In the end, in a differently induced model of PD, the repeated oral administration of *Proteus mirabilis* or its derived LPS to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated or young mice was sufficient to induce a PD-like phenotype with motor deficit, loss of dopaminergic neurons in the nigra, brain and gut inflammation with disruption of the intestinal epithelial barrier, and formation of α-syn inclusions in the brain and in the colon (185).

Neuropsychiatric Disorders

Neuropsychiatric disorders consist of cognitive, mental, and behavior disorders, such as schizophrenia, depression, anxiety, stress and bipolar disorders, autism, eating disorders, and epilepsy. In the last decades, the incidence of these conditions increased dramatically, reaching a percentage close to 40% of affected people worldwide. These patients have impaired health and ability to conduct a healthy life, to learn and work, which implies enormous health and economic effort from society. The etiology of these conditions includes genetic predisposition, injuries, infections, and environmental factors, such as the microbiota.

Patients suffering from major depressive disorder (MDD) had increased fecal α-diversity (increased levels of *Enterobacteriaceae* and *Alistipes* but reduced levels of *Faecalibacterium*) compared to drug-responders-MDD patients and healthy controls. The authors, therefore, reported a negative correlation between *Faecalibacterium* and the severity of depressive symptoms (186). An additional study suggested that the administration of probiotics (*Lactobacillus acidophilus, Lactobacillus casei*, and *Bifidobacterium bifidum*) to MDD patients significantly reduced depressive symptoms compared to placebo (187). From a large microbiome study on a Flemish population cohort, some bacteria have been associated with high quality of life, such as butyrate-producing *Faecalibacterium*, *Coprococcus* bacteria, and others with low quality of life and signs of

depression, such as Bacteroides enterotype 2 (22). From fecal metagenomic data, the bacterial capacity to synthesize 3,4-dihydroxyphenylacetic acid, a dopamine metabolite, correlates positively with mental quality of life and suggests a potential role of microbes to produce different neuroactive molecules during depression than during healthy conditions (22).

Patients affected by schizophrenia have also an altered and less rich gut microbiota composition with 77 differently expressed operational taxonomy units (OTUs) compared to healthy individuals (23). The fecal samples derived from these patients were transplanted into GF rodents and could transfer schizophrenic-associated behaviors, such as locomotor hyperactivity and decreased anxiety- and depressive-like behaviors, in the recipients (23). The mice that received fecal samples from schizophrenic patients showed several differentially regulated metabolic pathways in the feces, serum, and hippocampus (23). In particular, glutamine and GABA were elevated in the hippocampus (23). Glutamate was decreased in the stool and hippocampus of these mice, compared to mice transplanted with healthy patient's feces (23).

Regarding human autism spectrum disorder (ASD), studies showing the importance of the microbiota in the pathogenesis are few and mostly inconsistent, with some exceptions concerning the differences observed for bacteria, such as *Prevotella, Firmicutes, Clostridiales*, like *Clostridium perfringens*, and *Bifidobacterium* species (188), between the ASD patients and the controls. Colonizing GF mice with fecal microbiota from patients affected by ASD was sufficient to promote ASD-like behaviors in the animals (113). This approach seemed to be due to a deficit in the production of two bacterial metabolites, 5-aminovaleric acid (5-AV) and taurine, both being weak $GABA_A$ agonists, in ASD individuals compared to controls (113). Maternal immune activation (MIA) is a situation in which the maternal immune system gets activated by infections or infections-like stimuli, like LPS, and it features ASD in the offspring. In MIA animal models, scientists highlighted the importance of specific commensal bacteria in ASD protection. Interestingly, offspring coming from MIA dams showed intestinal microbial dysbiosis with 67 different OTUs compared to the control group, dysregulation of the intestinal barrier integrity with increased permeability, as reported already in children affected by ASD (189), and alteration in their metabolomic profile (20). The pure administration of two bacterial strains as probiotic treatment, such as *Bacteroides fragilis* (and *Bacteroides thetaiotaomicron* but not *Enterococcus faecalis*), could improve the gut dysbiosis, intestinal barrier integrity, the metabolic profile of the animals, and the communicative, repetitive, anxiety-like, and sensorimotor behaviors in the MIA model (20). Concerning the metabolites that could be induced by the intestinal bacteria under certain pathological conditions, in the MIA model, 4-ethylphenylsulfate (4EPS), indole pyruvate, serotonin, glycolate, imidazole propionate, and N-acetylserine were enormously increased in the serum of MIA offspring and entirely restored by the single *B. fragilis* treatment (20). Remarkably, the injection of the only 4EPS metabolite in the naïve healthy animals was sufficient to induce anxiety-like behaviors similar to the ones showed by the MIA offspring (20).

ROLE OF INFLAMMASOMES IN THE GUT-BRAIN AXIS: UNDER PHYSIOLOGICAL CONDITIONS

As mentioned earlier, in mice, the genetic deficiency of caspase-1, the effector molecule of the inflammasome, is associated with a decrease of innate and stress-induced depressive- and anxiety-like behaviors and affects chronic restraint stress response with a possible involvement of the intestinal microbiota (190).

The inflammasome is an innate immune signaling complex that is activated and assembled in response to the presence of pathogens or danger signals. Once activated, it leads to the production of active pro-inflammatory cytokines, such as IL-18 and IL-1β. There are several different inflammasomes. All consist of a "receptor" protein (such as pattern-recognition receptors (PRRs), like NLRs (NOD-like receptors) or TLR (Toll-like receptors), an adaptor molecule called apoptosis-associated speck-like protein (ASC or Pycard), and of the effector molecule pro-caspase-1. Additionally, inflammasome activation can initiate pyroptosis, a fast and pro-inflammatory form of cell death (106, 190, 191). In general, two signals initiate a successful inflammasome activation. The first signal comes from pathogen- or danger-associated molecular patterns (PAMPS/DAMPS) from outside of the cell and induces the transcription of genes encoding for inflammasome components and products. The second signal comes from intracellular danger signals, such as adenosine triphosphate, uric acid, fatty substances that can induce lysosomal damage, or nicotinamide adenine dinucleotide phosphate oxidase- or mitochondria-driven reactive oxygen species production. These processes result in the assembly and activation of inflammasomes (191–193).

In the CNS, inflammasome activation has been mainly linked to neuroinflammatory conditions. As example, it has an essential role in the progression of several neurological disorders like MS, AD, PPD, and NPS (132, 193–197). However, in the work of Wong ML et al., caspase-1–deficient mice were subjected to different behavioral tests, such as forced swim test, elevated plus maze, novelty suppressed feeding test, marble burying test, and open field test (190). Caspase-1–deficient mice, compared to wild-type mice, showed decreased floating time in the forced swim test, decreased anxiety-like behavior as measured by the unaltered open/closed arms time ratio in the elevated plus-maze (190). Latency to feed decreased after 16 hsec of fasting in the novelty suppressed feeding test (190). Caspase-1–deficient mice buried fewer marbles in the marble-burying test (190). In the open field test, the knock-out mice produced less fecal pellets, showed increased locomotion, and performed faster at the rotarod test (190). Upon treating the mice with either the minocycline antibiotic, which suppresses inflammasome activation and alters the microbiota composition, or after chronic restraint stress experiment for 21 consecutive days, the gut microbiota composition of these mice changed, compared with mice which do not undergo these procedures (190). The differences in microbiota composition resemble the ones reported in caspase-1–deficient mice, such as an increase in the relative abundance of *Lachnospiraceae* (198).

However, we would like to emphasize the fact that the influence of the host inflammasome pathways on the intestinal microbiota composition has been extensively discussed by colleagues and us (194, 199, 200). In fact, in different facilities, mice lacking ASC, NLRP6 (199), or NLRP3 (200) fail to show any detectable differences in the composition of their fecal flora compared to wild-type controlled littermates (199), either co-housed or individually housed, contrary to what has been described in other facilities (194). Instead, the maternal-microbiota inheritance or the cage effect had a much more substantial impact on the intestinal bacteria composition than the host inflammasome expression in our conditions (58). In conclusion, additional analyses are needed to understand in which particular hygiene conditions, mice lacking a functional inflammasome show remarkable microbiota alteration that could affect the health of the host. Moreover, IL-1β and IL-18 also have essential roles for physiological functions in the CNS, as they participate in processes of cognition, learning, and memory (201). Especially in the hippocampus and hypothalamus, high abundance of IL-1β has been described in steady-state in rats already in 1990, in regions of the brain conducting essential and conserved brain functions as memory and learning processes (202). In 1998, Schneider H. et al. could show that expression of IL-1β increases during long-term potentiation in neurons, which consists in a synaptic strengthening process implicated in learning and memory (203). However, in a study from 2004, IL-1β administration into the hippocampus induced memory impairments in a conditioning learning task in rats (204). This discrepancy underlies the complex role of inflammasomes and IL-1β in the CNS. Recently, AIM2 inflammasome has been shown to have a role in the normal brain development. It gets activated in presence of DNA damage that occurs at high levels during infections, trauma, but also neurodevelopment upon massive cell death (205). Under physiological conditions, AIM2 inflammasome is activated during neurodevelopment and contributes to CNS physiology acting through gasdermin-D regulation, and not through IL-1β and IL-18 production (205).

We could then speculate that in presence of certain infectious agents, or intestinal bacteria, the dysregulation of these physiological pathways could cause an overactivation of the inflammasome also in the brain and therefore an alteration of the homeostatic mechanisms. The disruption of these immune sensors could then lead to CNS abnormalities and disease conditions. Inflammasome signaling in the CNS has also been reported in the microglia, the brain's critical innate immune cells (206), in astrocytes (207), perivascular brain-resident macrophages (208), oligodendrocytes (209), endothelial cells (210), as well as in neurons (211). In the intestine, under physiological conditions, constant stimulation of inflammasomes is happening due to the resident trillions of microbes. It has been shown that released IL-18 importantly contributes to maintaining homeostasis in the gut (193). Several microbial factors can activate the intestinal inflammasome, which might have a distal effect on the brain. However, we still need to link all the events together. An evidence for intestinal inflammasome activation and its effects on the brain comes from the discovery that *Salmonella* leucine-rich repeat protein (SlrP) inhibits *Salmonella* virulence and the typical host anorexic response induced by the infection (212). SlrP inhibits the inflammasome activation and IL-1β production in the small intestine, preventing the flux of IL-1β to the hypothalamus *via* the vagus nerve and therefore the influence on the anorexic-feeding program in the CNS (212). It also promotes survival of the host, and from the microbial point of view microbial transmission to other hosts (212). Several microbial metabolites, such as taurine, spermine, and histamine, can affect the NLRP6 inflammasome activation in the gut and play an important role, if properly balanced, in host-microbial mutualism at the level of the intestine (213). Also, bile acids could be sensed by the inflammasome complex and induce their activation. Bile acids are produced in the liver conjugated with taurine or glycine and released in the upper small intestine during the digestion process. Once in the intestine, they can be converted by gut microbiota and affect the host metabolism and immune response. Researchers have identified that an analogue of oxo-12S-hydroxylithocholic acid methyl ester (BAA473), a microbiota-derived bile acid metabolite, the 11-12-oxo-lithocholic acid (BAA485) can induce the production of IL-18, in a pyrin-inflammasome-dependent manner (214). The group of MacKay C. showed that consumption of high-fiber diet, that inevitably has an effect on the host microbiota, or acetate-treatment, induced increased levels of IL-18 (and not IL-1β) in the serum of the animals *via* GPR43 and GPR109A receptors, that are expressed on colonic epithelial cells and activate the K^+ efflux and hyperpolarization of the cells and the Ca^{2+} mobilization, in an NLRP3- but not NLRP6-dependent manner, under both steady-state and inflammatory conditions (193).

These data show that intestinal inflammasome activation by the microbiota might lead to the production of effector molecules which have an effect in the CNS *via* the vagus nerve (212). This could be one of the innate immune pathway activated by the intestinal microbes with important distal effects also on the CNS, potentially during both healthy and inflammatory conditions.

ROLE OF INFLAMMASOMES IN THE GUT-BRAIN AXIS: UNDER PATHOLOGICAL CONDITIONS

An exact and overall understanding of the functions of inflammasome activation in physiologic and diseased states is of very high importance, as its effects are guiding to both situations and are tightly regulated. Following, we aim to summarize the state of research on the role of inflammasome activation in different CNS pathologies and what is known so far in terms of bacterial influence on the inflammasome pathways during CNS inflammation (**Table 1**).

Multiple Sclerosis

Inflammasome components and products have been shown to have a relevant role in MS pathogenesis. Indeed, Caspase-1 and ASC have recently been proposed as candidate biomarkers for MS onset (215). IL-1β and IL-18 seem to contribute to the

TABLE 1 | Inflammasome-dependent mechanisms in different neurological disorders.

Disorder/ Deficiency	Model	Treatment/ Method/ Genetically modified animals	Findings	Mechanism	References
Multiple sclerosis	MS patients	Analysis of serum samples	ASC and caspase-1 (inflammasome proteins) are potential biomarkers for prediction of disease onset ASC is a potential biomarker for severity of the disease.	ASC and caspase-1 were found to be elevated in the serum of MS patients compared to the control group. IL-1β was found to be decreased. ASC levels were higher in MS patients with moderate disease onset compared to the mild group.	Keane et al. (215)
Multiple sclerosis	MS patients		IL-1β, IL-18, Caspase-1↑ in PBMCs and the CSF.		Inoue and Shinohara (216); Mamik and Power 2017 (217)
EAE	Mouse	MOG-CFA immunization with pertussis toxin (PTX). IL-1RI$^{-/-}$ mice	IL-1 is importantly involved in the pathology of EAE by promoting pathogenic autoantigenspecific ThIL-17 cells, as IL-1RI-/- mice showed a lower incidence of EAE compared to wt mice after immunization.		Sutton et al. (218)
EAE	Mouse	MOG-CFA immunization with pertussis toxin (PTX)	Inflammasome activation in microglia and border-associated macrophages has a main responsibility for the development of EAE. Essential bacterial influence on the release of IL-1β.		Voet et al. (219)
EAE	Mouse	MOG-CFA immunization with enzymatically active and inactive pertussis toxin (PTX)	Inflammasome activation is needed for PTX-induced adhesion of neutrophils on cerebral capillaries and trigger encephalomyelitis in mice.	PTX activates TLR4 signaling in peritoneal myeloid cells, this leads to pro-IL-1β expression and the formation of a pyrin-dependent inflammasome, and active IL-1β. IL-1β stimulated stromal cells to secrete IL-6, which allows leukocyte adhesion on cerebral capillaries. Caspase-1-, ASC- or pyrin-deficient mice developed less severe EAE pathogenesis.	Dumas et al. (220)
EAE	Mouse	MOG-CFA immunization with pertussis toxin (PTX).Pycard$^{-/-}$ mice	Priming of encephalitogenic T helper CD4+ T cell subset was dependent on ASC-dependent IL-1β production.	PTX induced recruitment of monocytes and neutrophils into lymph nodes, where they produce IL-1β. This primes encephalitogenic T helper CD4+ cells which are responsible for the immune reaction and disease development. Priming of these T cells happens in an ASC-dependent production of IL-1β.	Ronchi et al. (113)
Alzheimer's Disease	AD patients		IL-1B, IL-18↑ in neurons, microglia and astrocytes surrounding Aβ plaques.		Griffin et al. (221), Simard et al. (222), Ojala et al. (223), Malaguarnera et al. (224), Öztürk et al. (225)
Alzheimer's Disease	AD patients		NLRP3, ASC, Caspase-1, -5, IL-1β, -18↑ in PBMCs.		Malaguarnera et al. (224), Saresella et al. (226), Bossù et al. (227)
Alzheimer's Disease	Primary mouse cells and cell lines for microglia	*In vitro* stimulation with Aβ amyloids	Fibrillar Aβ amyloids induced IL-1β secretion from microglia via NLRP3 inflammasomes		Halle et al. (228)

(Continued)

TABLE 1 | Continued

Disorder/ Deficiency	Model	Treatment/ Method/ Genetically modified animals	Findings	Mechanism	References
Alzheimer's Disease	Primary microglia	*In vitro* stimulation	NLRP3 activity spreads Aβ pathology in a prion-like manner promoting missfolded proteins to aggregate and form plaques.	Extracellular release of ASC particles (=ASC specks) from microglia cells function as danger signals. They bind to Aβ seeds which leads to further aggregation and spreading of Aβ plaques. ASC specks can also be inter- nalized by macrophages where they induce activation of caspase-1 and release of IL-1β.	Venegas et al. (229)
Alzheimer's Disease	Mouse	APP/PS1 mice deficient for Caspase-1, ASC or NLRP3	All these mice had reduced hippocampal and cortical amyloid plaques deposition with ameliorated disease outcome.	ASC-induced seeding leads to Aβ plaques aggregation and spreading. These ASC specks are released after immune activation of microglia.	Venegas et al. (229), Heneka et al. (230)
Alzheimer's Disease	Mouse	APP/PS1 mice. Pharmacological inhibition of NLRP3 inflammasome with MCC950	Aβ accumulation↓, inflammasome and microglia activation↓, neuroinflammation↓, cognitive impairments↓, phagocytotic function of microglia↑.		Dempsey et al. (231)
Alzheimer's Disease			Aβ accumulation activates microglia and promotes production of proinflammatory mediators by them, and also impairs their phagocytic function.		Sarlus and Hedeka (232), Shi and Holtzmann (233)
Alzheimer's Disease	Rat	IL-1β injection into cerebral hemisphere	Aβ-APP proteins overexpression and dystrophic neurite formation in the brain.		Sheng et al. (234)
Alzheimer's Disease	Mouse	APP/PS1 mice with sustained IL-1β↑↑ in the hippocampus	Plaques pathology↓		Shaftel (235)
Alzheimer's Disease	*in vitro* hiNSC-derived cell lines	HSV-1 viral infection	Induces gliosis and inflammation, production of IL-6, IL-1β, IFN-γ → low-grade HSV-1 infection induced an AD-like phenotype		Cairns et al. (236)
Tautopathy	Mouse	Tau 22 mice. Injection of brain homogenates of wt or APP/PS1 mice into the hippocampus of Tau22 mice.	Cleaved Caspase-1, ASC↑ in the brain at the age of 22 month compared to 3 months old Tau22 wt mice.		Ising et al. (197)
Tautopathy	Mouse	Tau 22 mice deficient for ASC or NLRP3. Injection of brain homogenates of wt or APP/PS1 mice into the hippocampus of Tau22 mice.	Tau hyperphosphorylation in CA1 region in Tau 22 wt mice but not in their $NLRP3^{-/-}$ and $ASC^{-/-}$ counterparts. → NLRP3 activation is upstream of Aβ-tau cascade and pathology, in a IL-1β-dependent manner.		Ising et al. (197)
Tautopathy	Mouse	ASC-, Caspase-1-, IL-1R-, Myd88-deficient mice. Injection of fibrillar Aβ into the striatum.	NALP3 inflammasome and the IL-1β pathway is essential for the microglia activation upon Aβ deposition in the brain.	↓Recruitment and activation of microglia and phagocytes to the Aβ injection site in brain of these mice compared to wt mice.	Halle et al. (228)

(Continued)

TABLE 1 | Continued

Disorder/ Deficiency	Model	Treatment/ Method/ Genetically modified animals	Findings	Mechanism	References
Parkinson's Disease	PD patients		IL-1β, Caspase-1↑ in the serum and striatum		Mogi et al. (237)
Parkinson's Disease	PD patients and rodents		Fibrillar form of α-synuclein induced NLRP3- and caspase-1-mediated IL-1β secretion in monocytes and BV2 microglia, or via TLR2-signaling in microglia		Codolo et al. (238)
Parkinson's Disease	PD patients and mice	Patients with a PARK2 mutation, PARK2-/-, PINK1-/- mice	Exacerbation of NLRP3 inflammasome activation in microglia and macrophages in PARK2-/- and PINK1-/- mice. This was confirmed in blood-derived macrophages of patients with PARK2 mutation.		Mouton-Liger et al. (239)
Parkinson's Disease	Human dopaminergic neuroblastoma cells		active caspase-1 cleaves α-synuclein which then newly forms α-synuclein aggregates. Neuronal toxicity↑		Wang et al. (240)
Parkinson's Disease	Rat	Intranigral injection of IL-1β with adenoviruses	Chronic expression of IL-1β in substantia nigra induced activation of astrocytes and microglia and progressive death of dopaminergic neurons → motor impairments.		Ferrari et al. (241)
Parkinson's Disease	Rat	LPS-induced and 6-hydroxy-dopamine-induced PD rats. Injection of caspase-1-inhibitor Ac-YVAD-CMK	Inhibition of caspase-1 → NLRP inflammasome signaling proteins↓ and improvment in the number of dopaminergic neurons.		Mao et al. (242)
Parkinson's Disease	Mouse	α-synuclein A53T transgenic PD mice deficient for caspase-1	IL-1β levels in the midbrain↑. Caspase-$1^{-/-}$ → activation of microglia↓		Fan et al. (243)
Parkinson's Disease	Mouse	MPTP-induced PD mice deficient for NLRP3	Dysregulation of NLRP3 inflammasome contributes to the development of MPTP-induced loss of nigral dopaminergic neurons. Circulating dopamine is a NLRP3 inflammasome inhibitor.	NLRP3-/- mice are resistant to the loss of dopaminergic neurons induced by MPTP. This was associated with caspase-1-, IL-1β- and IL-18↓	Yan et al. (244)
Neuropsychiatric disorders	Depressed, bipolar, and ASD patients		↑expression and activity of NLRP3 inflammasome		Alcocer-Gómez et al. (245) Saresella et al. (226) Baroja-Mazo et al. (246)
Neuropsychiatric disorders	MDD patients		↑ NLRP3, caspase-1, IL-1β mRNA and proteins in PBMC		Alcocer-Gómez et al. (247)
Neuropsychiatric disorders	SSD, ASD, OCD, NSSID patients		↑ NLRP3, caspase-1, ASC, IL-1β, IL-1RN, TNF mRNA in PBMC, ↑plasmitic levels of IL-1↑, IL-18, IL-1Ra, TNFα, and IL-6		Hylén et al. (248)

(Continued)

TABLE 1 | Continued

Disorder/ Deficiency	Model	Treatment/ Method/ Genetically modified animals	Findings	Mechanism	References
Neuropsychiatric disorders	MDD patients	Antidepressant drugs	Drugs have inhibitory effect on NLRP3-inflammasome activation, reduction in serum levels of IL-1β and IL-18 and protein levels of NLRP3 and IL-1β		Alcocer-Gómez et al. (247)

pathophysiology of MS, as they are upregulated together with caspase-1 in peripheral blood mononuclear cells (PBMCs) and the cerebrospinal fluid (CSF) of MS patients (216, 217).

Evidences in animal models also revealed the importance of the inflammasome signaling in microglia and border-associated macrophages during EAE (219). Essential bacterial influence on the release of IL-1β in the classical animal model of active induced EAE was shown by others and us a few years ago (132, 218, 220). In fact, the importance of the usage of pertussis toxin (PTX) to induce the disease development in an mouse model for MS started to be elucidated. PTX is the major virulence factor of *Bordetella pertussis* and needs to be used in conjugation together with the antigen and the adjuvant during immunization. Dumas A. and colleagues reported the effects of PTX-induced IL-β in the recruitment of inflammatory leukocytes into the brain upon upregulation of adhesion molecules on blood–brain capillaries (220). Briefly, PTX, with its ADP-ribosyltransferase active subunit, induced the activation of TLR4 signaling in peritoneal myeloid cells (macrophages and neutrophils), pro-IL-1β expression, and therefore the formation of a pyrin-dependent inflammasome that releases active IL-1β (220). Subsequently, IL-1β stimulated the stromal cells to secrete IL-6, which is known to induce vascular changes required for leukocyte adhesion. In caspase-1-, ASC-, or pyrin-deficient hosts, PTX did not induce neutrophil adhesion to cerebral capillaries and therefore leads to a less severe EAE phenotype (220). In addition, we showed that expression and production of IL-1β (and not IL-18, IL-1α, IL-6, or IL-23) was transiently and shortly increased post-PTX (and not PBS) injections, in the draining lymph-nodes, during the priming phases of the disease model (132). This happened earlier than the appearance of any clinical symptom (132). The toxin induced the recruitment of inflammatory monocytes and neutrophils in the draining lymph-nodes which were responsible of the production of IL-1β in the tissue (132). IL-1β–producing lymphoid myeloid cells were needed for the priming of multifunctional encephalitogenic T cells, characterized by the production of IL-17A, IFN-γ, GM-CSF, and IL-22 (132). The priming of this pathogenic subset of T helper $CD4^+$ T cells was dependent on ASC-dependent IL-1β production, and not on IL-12p35 or IL-12p40 (132). Interestingly we have also shown that the signaling of IL-1β both at the level of T and non-T cells was necessary to generate the immune reactions involved in the disease development (132). We therefore suggested that environmental (bacterial) factors can also affect the priming of autoreactive pathogenic T cells providing new insights into the pathogenic mechanisms of MS and other immune-mediated diseases, including neurological disorders.

In summary, the infectious agent products could be crucial in the activation of the inflammasome pathway in MS pathogenesis. Hence, it would be intriguing to study if similar events could be driven by mucosal commensal microbes in order to better manipulate the microbiota and the immune mediated disease pathogenesis.

Alzheimer's Disease

Inflammasome and its products have been implicated in AD pathogenesis since a higher expression of IL-1β and IL-18 has been reported in the microglia, astrocytes, and neurons that surround Aβ plaques or in the plasma of AD patients (221–225). The higher expression of NLRP3, ASC, caspase-1, caspase-5, IL-1β, and IL-18 was additionally found in the PBMCs of AD patients (224, 226, 227). In general, patients affected by tauopathies showed elevated levels of cleaved caspase-1 and ASC and mature IL-1β in the cortex (197). NLRP3 inflammasome-mediated neuroinflammation has been importantly implicated in pathogenesis and progression of AD. Fibrillar Aβ amyloids can induce the secretion of IL-1β from microglia, *via* the NLRP3 inflammasome (228, 249). In turn, NLRP3 inflammasome activity leads to the extracellular release of ASC particles that may function as danger signals (246). They were shown to physically bind to Aβ, seed, and then spread Aβ pathology in a prion-like manner by promoting misfolded proteins to aggregate and form plaques (229).

Animal studies confirmed this significant involvement of NLRP activation, as APP/PS1 mice, lacking the expression of ASC, caspase-1, or NLRP3 had significantly reduced hippocampal and cortical amyloid plaque deposition and ameliorated disease outcome (229, 230). Also, Tau22 mice, another model of AD and other tauopathies, had increased levels of cleaved caspase-1 and ASC in their brain at the age of 11 months compared to their 3-month-old counterparts. Consistent with this, Tau22 mice lacking ASC or NLRP3 expression had lower levels of aggregated and hyperphosphorylated tau in the hippocampus, and their typical spatial memory deficits were rescued (197). Injecting brain homogenates from APP/PS1 or wild-type mice into the hippocampus of Tau22 mice, induced tau hyperphosphorylation in the hippocampus of Tau22 wild-type but not Tau22/$Pycard^{-/-}$ or Tau22/$Nlrp3^{-/-}$ deficient mice, suggesting that NLRP3 activation is upstream the Aβ-tau cascade and tau pathology (197). Moreover, NLRP3 activation induces tau hyperphosphorylation and aggregation in an IL-1β–dependent manner. Importantly, researchers also found that ASC-, caspase-1-, IL-1 receptor-, and

MyD88-deficient mice had less recruitment of microglia and mononuclear phagocytes to Aβ in the brain as compared to the wild type control after the injection of Aβ into the striatum (228).

In addition, the pharmacological inhibition of the NLRP3 inflammasome reduced Aβ deposition, neuroinflammation, and cognitive impairment in the APP/PS1 AD mouse model (231). Aβ accumulation activates the microglia and promotes pro-inflammatory mediators' production and impairment of their phagocytic function (232, 233). IL-1β injection into the cerebral hemisphere increases Aβ-APP proteins in wild-type rats (234). All these evidences suggest that the inflammasome activation in AD pathogenesis could be downstream the Aβ deposits formation, and it could then amplify the neuroinflammation linked to the disease. However, IL-1β seems to have an intricate role, since a sustained overexpression in the hippocampus in APP/PS1 mice was shown to reduce plaque pathology (235). A very recent study highlighted the casuality of *Herpes simplex virus type 1* (HSV-1) in the AD development (236). The authors showed that infecting human-induced neural stem cell (hiNSC) lines with HSV-1 *in vitro* induced gliosis and inflammation, including the production of pro-inflammatory cytokines such as IL-1β, IL-6, and IFN-γ. This work, furthermore, showed that low-grade HSV-1 infection induced an AD-like phenotype in brain organoids derived from hiNSCs (236).

Parkinson's Disease

Increased IL-1β and caspase-1 have been measured in the serum and the striatum of PD patients (237). The fibrillar, but not monomeric, form of α-synuclein induced NLRP3- and caspase-1–mediated IL-1β secretion in human monocytes and BV2 microglial cell line (238), or *via* TLR-2 signaling pathway in rodents microglial cells (250). Additionally, early-onset PD patients with mutations in two genes encoding for parkins (PARK2 and PARK2), or mice lacking a mitochondrial serine/threonine protein kinase (PINK1) showed an exacerbated NLRP3 inflammasome response in their microglia and macrophages (239).

In animal models, active caspase-1 was shown to directly cleave α-synuclein, which further promoted the aggregation and neuronal toxicity for neurons of this newly-aggregated α-synuclein (240). Moreover, the chronic expression of IL-1β in substantia nigra of rats induced progressive death of dopaminergic neurons and resulted in motor impairments (241). Injection of the caspase-1 inhibitor Ac-YVAD-CMK was shown to reduce the expression of NLRP inflammasome signaling proteins and improve the number of dopaminergic neurons in LPS- and 6-hydroxydopamine-induced PD in rats (242). In the α-synuclein A53T transgenic mouse model of PD (which overexpress the mutant human A53/α-synuclein), elevated levels of IL-1β in midbrain were measured, but when the mice lack the endogenous expression of caspase-1, this significantly reduced the activation of microglia (243). Moreover, NLRP3$^{-/-}$ mice were resistant to the loss of nigral dopaminergic neurons induced by treatment with the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), and this was associated with a reduction in caspase-1 activation and IL-1β and IL-18 secretion (244). However, also for PD, it is very unclear if any microbial insult could be the trigger of the deposit formation that induce the inflammasome activation or vice versa (251, 252).

Neuropsychiatric Disorders

Increased expression and activity of the NLRP3 inflammasome machinery in circulating immune cells of patients affected by depression (245), bipolar disorder (246), and ASD (226), have been reported. However, works aimed to study how the host system changes in these types of psychiatric disorders are still lacking. A study involving a relatively small cohort of MDD patients and healthy volunteers reported that NLRP3 mRNA and protein levels are increased in peripheral blood mononuclear cells in MDD patients, as caspase-1 and IL-1β, and normalized to healthy levels upon antidepressant treatment (245). In a recent work, involving 40 psychiatric patients affected by schizophrenia spectrum disorders (SSD) (including psychotic disorder, schizophrenia, and schizoaffective disorder), ASD, obsessive-compulsive disorder (OCD), or non-suicidal self-injury disorder (NSSID), expression of genes encoding for NLRP3, caspase-1, ASC, IL-1β, IL-1RN, and TNF are significantly increased in peripheral whole blood of psychiatric patients compared to matched healthy controls (248). Also, at the protein level, the amount of plasma IL-1β, IL-18, IL-1Ra, TNF-α, and IL-6 were more elevated in the psychiatric patients compared to the healthy controls (248). In details, patients with SSD had higher levels in IL-18, IL-1Ra, TNF, and IL-6; whereas OCD patients had higher levels of IL-18, IL-1Ra, and TNF, compared to the healthy controls (248). Importantly, these effects were not caused by the presence of functional mutations of inflammasome components or products, which could lead to increased inflammasome activity and cytokine release, neither by the body mass index (BMI), age at disease onset, depression, or treatment with psychotropic drugs (248). Antidepressant drugs can have an inhibitory effect on inflammasome activation, especially on NLRP3 inflammasome, as they reduce serum levels of IL-1β and IL-18 and protein levels of NLRP3 and IL-1β (247). Host metabolites have an inhibitory effect on NLRP3 inflammasome followed by anti-inflammatory cascade and beneficial effect on the brain, with antidepressant action as example. Specifically, the β-hydroxybutyrate (BHB), a physiological ketone body produced by the liver in condition of fasting, low blood sugar, or carbohydrate-free (like ketogenic) diet consumption had an inhibitory effect on NLRP3-inflammasome (253). In rats, repeated subcutaneous injections of BHB attenuated stress-induced IL-1β and TNF-α expression in the hippocampus. The release of IL-1β and TNF-α caused by stress is tightly regulated by NLRP3 inflammasome (254). These findings suggest that BHB exerts antidepressant-like effects, possibly by anti-inflammatory mechanisms that inhibits or are led by NLRP3-induced neuro-inflammation in the hippocampus (255). This study could also suggest a possible future therapeutic usage of metabolites like BHB to treat neuropsychiatric disorders such as stress-related mood disorders.

SUMMARY AND OPEN QUESTIONS

Emerging data about the influence of intestinal inflammation on the nervous system is extremely critical to connect the missing dots between the two organs for better understanding the synergistic communication within the GBA. This review aimed to present how certain bacterial species could shape the host GBA during healthy and disease conditions. Then, it centralized the attention on the known mechanisms of action of bacteria through the production of molecules that can influence the host's immune and nervous functions. However, in many cases, in both patients and disease animal models, the exact mechanisms of action and signaling pathways activated by the bacteria and their products or metabolites are yet to be discovered. We also presented how the innate immune inflammasome pathway could act, in some cases, as communication tool between the microbes and the CNS, however still many questions are unanswered. Future studies aiming to dissect the exact mechanisms of microbial action are of critical urgency in the field. Translational approaches and more significant clinical trials are utterly needed to understand the temporal and causal relationships between gut microbiota and specific disease development, to evaluate the suitability of the microbiome as a biomarker of disease, and the efficacy of microbial interventions, such as probiotics and FMT protocols, in the patients. Clinical studies, coupled with animal experiments, are needed to precisely dissect the molecular pathways behind the pathogenesis of several disease conditions. This approach would be necessary for examining the mechanisms behind a specific immunological or neurological effect observed in the presence or absence of particular bacterial species in certain pathogenic conditions. In this case, this review highlights the necessity of studies aimed to discover if and how individual bacterial molecules (products or metabolites) are involved in disease progression or protection. We believe that future research in the field must aim to reveal the precise mechanisms behind the pro- and anti-inflammatory responses induced by the microbes. Of crucial relevance will also be to understand what specific bacterial molecules and metabolites exactly do at the CNS level and how they reach the CNS. It is vital to study on which cells they act, which signaling pathways they activate or suppress, in which organ these mechanisms are affected, and whether they affect both the enteric and central nervous systems. Additionally, it will be essential to address how alterations of the host immune or nervous system will affect also the functions of the microbiota, *via* inflammatory mediators and defensive molecules.

The inflammasome is a signaling pathway that might be activated in the presence of certain bacteria and bacterial molecules. As shown, it is involved in several neurological and intestinal homeostatic and inflammatory conditions. Inflammasome products are targets of several therapies used to treat some of the disorders that we have presented here (256). In some cases, it is involved in the pathogenesis of neurodegenerative diseases, such as EAE, upon bacterial exposure (132). However, it is still obscure if and how intestinal microbial alterations, which are associated with every neurological disease, are upstream or downstream of the immunological (like inflammasomes) and neurological dysfunctions. It is necessary to dissect in which microbial conditions specific mechanisms are activated and how. This approach will allow to design more efficient therapies aimed to modulate the microbiota or the host immune responses to ameliorate or cure specific neurological pathologies.

Overall, we think that the exciting and important discoveries here summarized suggest that bacteria, both pathogens and commensals, have the capacity to stimulate the host intestinal tissue and signal to the brain to promote several aspects of the behaviors of the host and the neurological disease pathogenesis. It is now historically the stage in which all the tools and instruments to identify single bacteria and their products are available. It is possible to then follow them in the various host tissues to understand where they go, which cells they can affect, and which pathways they can activate. This mechanistic approach is, at the moment, utterly needed to better understand how the nervous system is influenced by the intestine. We believe that this knowledge will also lead to the understanding of how to develop better interventions and more efficient and personalized therapeutic strategies for patients affected not only by the neurological disorders treated in this review.

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AR and FR wrote the manuscript and generated figures and tables. JK generated figures. All authors contributed to the article and approved the submitted version.

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MINI REVIEW
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The Joint-Brain Axis: Insights From Rheumatoid Arthritis on the Crosstalk Between Chronic Peripheral Inflammation and the Brain

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Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by erosive polyarthritis. Beyond joint pathology, RA is associated with neuropsychiatric comorbidity including depression, anxiety, and an increased risk to develop neurodegenerative diseases in later life. Studies investigating the central nervous system (CNS) in preclinical models of RA have leveraged the understanding of the intimate crosstalk between peripheral and central immune responses. This mini review summarizes the current knowledge of CNS comorbidity in RA patients and known underlying cellular mechanisms. We focus on the differential regulation of CNS myeloid and glial cells in different mouse models of RA reflecting different patterns of peripheral immune activation. Moreover, we address CNS responses to anti-inflammatory treatment in human RA patients and mice. Finally, to illustrate the bidirectional communication between the CNS and chronic peripheral inflammation, we present the current knowledge about the impact of the CNS on arthritis. A comprehensive understanding of the crosstalk between the CNS and chronic peripheral inflammation will help to identify RA patients at risk of developing CNS comorbidity, setting the path for future therapeutic approaches in both RA and neuropsychiatric diseases.

Keywords: rheumatoid arthritis, neurodegenenerative diseases, depression, blood-brain barrer, microglia, neuroinflammation

INTRODUCTION

The central nervous system (CNS) has long been considered to be protected from circulatory inflammatory signals by the blood-brain barrier (BBB). However, an intimate crosstalk between chronic peripheral inflammation and the CNS is evidenced by a plethora of neurological and psychiatric sequelae associated with chronic inflammatory diseases like rheumatoid arthritis (RA).

RA is a systemic autoimmune disease characterized by synovial inflammation and deformation of joints and adjacent bones. The pathogenesis of RA is driven by a complex interplay between the adaptive

immune system involving T-cells and autoantibodies as well as innate immune components like myeloid cells and pro-inflammatory cytokines (1–3). RA patients are highly predisposed to develop neuropsychiatric comorbidities. The prevalence of major depressive disorder in RA patients was estimated to be 16.8%, by far exceeding the general population (4, 5). Moreover, RA patients show higher levels of anxiety (6) and impaired cognitive performance (7, 8) compared to healthy individuals. Additionally, almost 40% of RA patients experience chronic pain, which is further linked to depression and anxiety (9). Interestingly, mid-life RA lead to an increased risk to develop dementia by 2.5-fold after a follow-up period of 21 years (10). However, epidemiological studies on the association between RA and individual neurodegenerative diseases like Alzheimer's Disease (AD) and Parkinson's Disease (PD) showed contradictory results (11–16). Nevertheless, RA therapeutics inhibiting pathogenetic pro-inflammatory cytokines like tumor necrosis factor (TNF) and interleukin-6 (IL-6) alleviate symptoms such as depression and anxiety (17, 18) and were linked to a reduced risk of future neurodegeneration (19) in RA patients.

While precise pathological mechanisms causing CNS involvement in RA are currently being investigated, most existing insights about the propagation of peripheral inflammation into the CNS and subsequent impairment of neural function are derived from animal models of acute infection by administration of lipopolysaccharide (LPS) or polyinosinic-polycytidylic acid (Poly(I:C)). In this context, peripheral inflammatory mediators can enter the CNS across the BBB or the choroid plexus, by the infiltration of blood-derived immune cells or the inflammatory activation of endothelial cells (20–23). Additionally, astrocytes and CNS-associated myeloid cells like parenchymal microglia, meningeal, perivascular and choroid plexus macrophages, acquire an inflammatory state. These changes are referred to as "neuroinflammation" and may ultimately be the link to neuropsychiatric symptoms by inducing neuronal damage, impaired adult hippocampal neurogenesis, and altered neurotransmitter signaling (20, 24–26).

Transferring these findings into the context of chronic peripheral inflammation is urgently needed as neuropsychiatric comorbidity in RA substantially contributes to disease burden and worsens therapeutic response and outcome (27, 28). However, research on this topic is hampered by the heterogeneity of present experimental models (29) and immunophenotypes observed in RA patients (30–32). The present review summarizes our current knowledge about inflammatory alterations and neuronal dysfunction in the brain of RA patients and rodent models. Vice versa, we will also discuss how the CNS is able to modulate the course of peripheral arthritis. Additionally, we aim to highlight open questions and future research strategies to better decipher and treat neuropsychiatric vulnerability in chronic peripheral inflammation.

GENETIC LINKS BETWEEN CHRONIC INFLAMMATION AND NEURODEGENERATION

The clinical CNS involvement of some patients with RA has raised the question of a shared genetic predisposition for RA and neurological or psychiatric diseases. A particular focus was aimed towards neurodegenerative diseases, since the immune system is increasingly being acknowledged as an important driver of pathogenesis. Recently, a comparison of genome-wide association studies (GWAS) on neurodegenerative and chronic immune-mediated diseases revealed 15 single-nucleotide polymorphisms (SNPs) jointly associated with RA and frontotemporal dementia (FTD). Interestingly, the majority of those SNPs were located in the human leukocyte antigen (HLA) region on chromosome 6 (33). This region encodes a set of gene products essential for self- and non-self-antigen presentation and immune function both in the periphery and the CNS. The dense and overlapping organization of HLA-genes on chromosome 6 did not allow the identification of individual genes accounting for the shared risk between FTD and RA. Shared disease-associated SNPs related to immune function were also identified for RA and PD (34), amyotrophic lateral sclerosis (ALS) and progressive supranuclear palsy (PSP) (33). Yokoyama et al. identified few SNPs jointly associated with AD and different immune diseases including RA (35). In contrast, Mendelian Randomization studies showed no positive correlation between known genetic risk factors for RA and incidence of AD (15, 36) or vice versa (37). Interestingly, the polygenic risk for RA integrating many known predisposing SNPs was correlated with lower cognitive performance in healthy adolescents (38). Felsky et al. demonstrated a correlation between polygenic risk for RA and microglial density in the brain of elderly individuals (39). Again, this correlation was substantially driven by genetic changes located in the HLA region on chromosome 6 (39). Taken together, HLA-associated polymorphisms and immune-related genetic risk factors for RA may contribute to comorbid cognitive impairment and neurodegenerative diseases.

ROUTES CONVEYING CHRONIC PERIPHERAL INFLAMMATION TO THE BRAIN

To induce neuroinflammation in the context of arthritis, peripheral inflammatory signals must enter into the CNS. This is achieved by several routes (**Figure 1**). First, circulatory pro-inflammatory cytokines are able to enter the brain by volume diffusion in circumventricular organs (CVOs), neuroanatomical sites of increased BBB permeability located around the third and fourth ventricles (20). Besides, studies based on LPS-induced acute peripheral inflammation proposed the entrance of cytokines into the brain by active transport or tight junction damage (40, 41). In the context of RA, disruption of the BBB was observed in a collagen-induced arthritis (CIA) model (42, 43). This model is based on immunization against collagen-II and strongly driven by T-cell-dependent mechanisms. Correspondingly, brain homogenates of CIA-induced mice showed an increased cell population expressing monocyte markers C-C motif chemokine receptor 2 (Ccr2) and Ly6c (44). This finding might indicate blood-derived myeloid cell infiltration into the brain parenchyma, although perivascular or meningeal localization of the detected cells was not excluded. In contrast to CIA, BBB tight junctions remained intact in mice

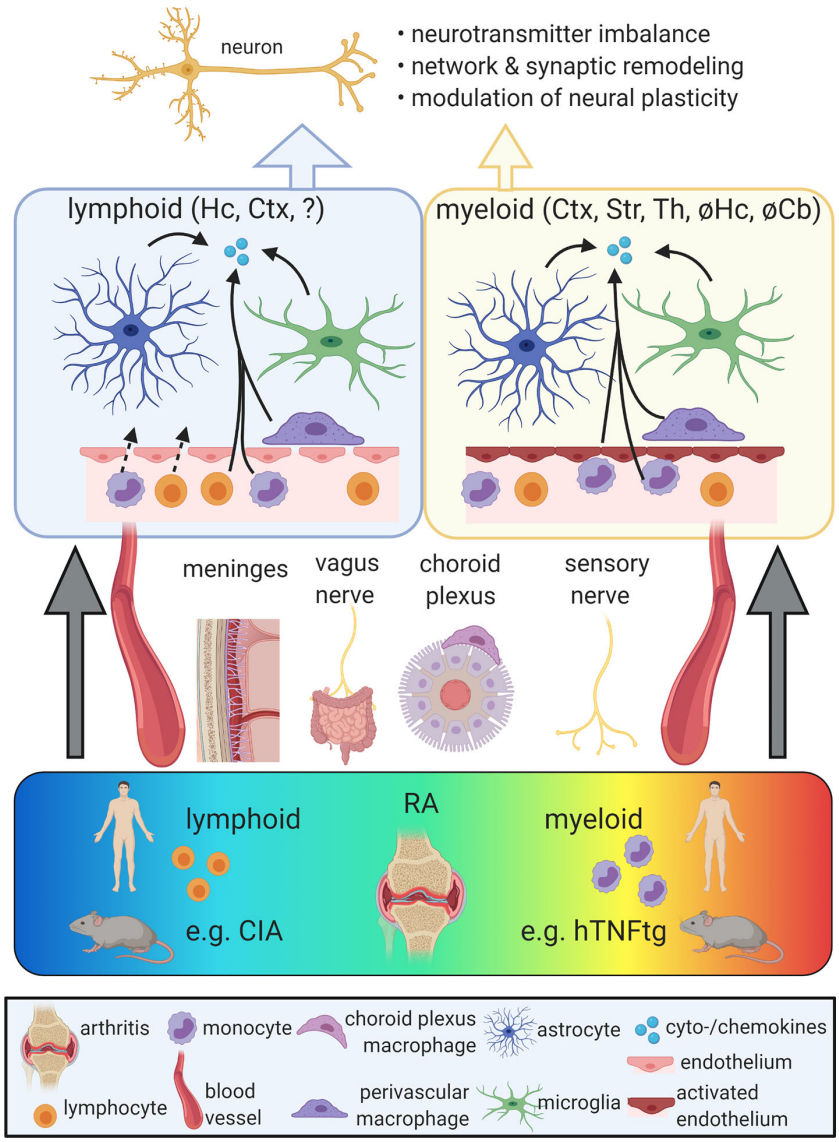

FIGURE 1 | Propagation of chronic peripheral inflammation into the central nervous system (CNS). Rheumatoid arthritis (RA) comprises a spectrum of different peripheral immunophenotypes, including a lymphoid subtype driven by adaptive immune activation and a myeloid subtype characterized by the activation of myeloid cells. While the lymphoid subtype is represented by the mouse model of collagen-induced arthritis (CIA), the human TNF-α transgenic (hTNFtg) mouse model mimicks key aspects of the myeloid form of RA. Peripheral inflammation can reach the CNS *via* different gateways, including the vagus nerve, the somatosensory nervous system, the meninges, the choroid plexus and the bloodstream. In both lymphoid and myeloid models of RA, the activation of microglia, astrocytes, and perivascular macrophages as well as increased levels of pro-inflammatory cytokines and chemokines were described. While in CIA, these alterations were mainly observed in the cortex (Ctx) and hippocampus (Hc), hTNFtg mice show a distinct regional distribution of neuroinflammation including the Ctx, striatum (Str), and thalamus (Th), but sparing the Hc and the cerebellum (Cb). The blood-brain barrier (BBB) was proposed to be disrupted in lymphoid models, potentially allowing the influx of blood-derived immune cells. In myeloid models, BBB integrity appears maintained, but endothelia display an activated signature and may contribute to the secretion of cytokines and chemokines. Neuroinflammation in RA models was linked with impaired neuronal function due to altered neurotransmitter metabolism and neural plasticity as well as synaptic and network refinement. Ultimately, these changes may cause neuropsychiatric symptoms. So far, behavioral phenotypes were mainly found in lymphoid models of RA and are limited in the myeloid subtype. Figure created with BioRender (https://biorender.com).

overexpressing human TNF in the periphery (hTNFtg) (45), a model characterized by a profound myeloid cell activation without T-cell involvement (46, 47). In line with BBB integrity in this model, single cell RNA-seq of cortical myeloid cells showed no increase in the number of blood-derived monocytes or granulocytes (45). In summary, findings on BBB disruption differ between RA animal models and evidence for CNS infiltration of peripheral myeloid cells in RA is limited. The role of T cells in the CNS involvement of RA is largely unaddressed.

Peripheral inflammation may also induce non-disruptive alterations of the BBB, including an upregulation of leukocyte adhesion markers and the secretion of inflammatory mediators

by endothelial cells (48). In hTNFtg mice, intercellular adhesion molecule 1 (Icam-1) and vascular cell adhesion molecule 1 (Vcam-1) are induced in distinct brain regions, indicating endothelial activation without tight junction leakage (45). Interestingly, endothelial Vcam-1 was recently described to mediate neuroinflammation and cognitive impairment during aging. This process was accompanied by intravascular adhesion, but not parenchymal infiltration of circulatory leukocytes (49). In Complete Freud's Adjuvant-induced arthritis, endothelia contribute to vessel-associated micro- and astrogliosis and subsequent hyperalgesia by vascular endothelial growth factor 2 (Vegf2)-dependent upregulation of Icam1 in the spinal cord (50). Hence, BBB endothelial cells may act as an active mediator of neuroinflammation rather than a passive barrier in the context of RA.

Moreover, chronic peripheral inflammation is propagated into the CNS by direct neuronal routes. Pro-inflammatory cytokines like IL-1β, IL-6, and IL-17 were shown to activate peripheral nociceptive afferents (51). These neuronal afferents are involved in the pathogenesis of arthritis, but also signal inflammatory cues to the CNS. Accordingly, Hess et al. demonstrated a profoundly increased pain response in the brains of hTNFtg mice and RA patients by functional magnetic resonance imaging (fMRI), which was reversible upon inhibition of peripheral TNF (52). This modulation of CNS activity preceded the histopathological amelioration of arthritis (52). Apart from the somato-afferent nervous system, the vagus nerve may display a second neuronal afferent route linking chronic peripheral inflammation and the CNS. The vagal nerve is involved in the generation of behavioral responses to LPS administration (53) and activated by TNF and IL-1β in cytokine-specific electrophysiological patterns (54). Future experiments transferring these findings into the context of arthritis are needed.

Finally, further interfaces between the peripheral immune system and the CNS, including the choroid plexus, the meninges and the glymphatic system have hardly been studied in chronic peripheral inflammation and might provide further insights into the involvement of the CNS in RA. Moreover, gut microbiota can modulate microglia and brain function (55, 56). As gut dysbiosis was shown in RA (57), the gut-brain axis might as well contribute to RA-associated neuropsychiatric comorbidity.

NEUROINFLAMMATION IN RA

After receiving inflammatory signals from the periphery, CNS-resident cells, particularly microglia and astrocytes, are able to acquire an activated phenotype and maintain a neuroinflammatory state. Recently, we characterized myeloid cells in hTNFtg mice by histology, flow cytometry, and scRNA-seq. We detected a strong microglial activation signature with a downregulation of homeostatic markers like transmembrane protein 119 (Tmem119), P2ry12, and Fc receptor-like S (Fcrls) accompanied by the upregulation of CD45, sialic acid-binding immunoglobulin-type lectin 1 (Siglec-1), several complement factors, and chemokines as well as genes linked to lysosomal function (45). Importantly, this microglial response was restricted to defined brain regions, including the cortex, striatum, and thalamus, and reversed by inhibition of peripheral human TNF using infliximab, a clinically used compound for the treatment of RA (45). In the cerebellum and hippocampus of hTNFtg mice, there was little, if any, inflammatory response. This regional vulnerability of the CNS may be linked to locally confined endothelial activation (45). The low susceptibility of the hippocampus, a region involved in the pathophysiology of depression and memory disorders, may explain the absence of depressive-like symptoms in hTNFtg mice (58). When these mice were crossed with the 5XFAD model of AD, decreased amyloid plaque load was detected. However, the authors also suggested impaired synaptic integrity, which might be due to an unselectively boosted phagocytosis of activated myeloid cells (59). Behavioral analyses are needed to answer the question if TNF-driven arthritis overall ameliorates or worsens AD-like phenotypes in 5XFAD mice.

In contrast to microglia, the response of astrocytes is poorly characterized in TNF-driven arthritis and only based on the expression of glial fibrillary acidic protein (Gfap). While in hTNFtg mice, reactive astrogliosis was concluded by increased Gfap staining intensity in the cortex (59), arthritis driven by overexpression of murine TNF was associated with activation of microglia, but not astrocytes (60).

In contrast to TNF-driven arthritis, lymphoid cell-based models of RA like CIA or antigen-induced arthritis (AIA) were less extensively investigated regarding neuroinflammation, mainly focussing on the hippocampus and cortex. Several studies reported an elevated expression of TNF, IL-1β, or IL-6 in the hippocampus and cortex of arthritic mice (43, 44, 61, 62). Moreover, microglial density and expression of the phagocytosis marker CD68 were increased in the hippocampus during CIA (43, 61). Increased phagocytosis was corroborated by a decrease of amyloid or tau pathology, when CIA was induced in AD mouse models (43, 44). However, analyses of neuronal integrity and behavior were not performed in these studies. Besides activation of microglia, CIA led to increased numbers of $Gfap^+$ astrocytes in the hippocampus (61). Hippocampal inflammation in CIA and AIA was associated with depressive-like behavior (61, 62).

Together, these data show neuroinflammation in immunization-based RA models. Interestingly, hippocampal immune response in these models indicates a different regional pattern of neuroinflammation compared to myeloid cell-based, TNF-driven arthritis. It is therefore tempting to speculate, that the peripheral immunophenotype during chronic inflammation navigates regional neuroinflammation. Importantly, several recent transcriptomic studies point out the existence of different immunophenotypes in RA patients (30–32). This includes phenotypes with lymphoid-based and myeloid-based inflammation (30, 32), which reflects the pathophysiological hallmarks of CIA and the hTNFtg model, respectively. Thus, a comparison of BBB alterations, neuroinflammation, and neuropsychiatric comorbidity between these different subtypes of RA patients might help to understand predisposing factors for neurological or psychiatric comorbidity.

In RA patients, neuroinflammation has hardly been examined. Analyses of human *post mortem* brain tissue indicated microglial activation evident by downregulation of the homeostatic marker

P2RY12 in the cortex, but not the cerebellum of RA patients. This finding matches with the regional CNS immune response previously observed in hTNFtg mice (45). The cerebrospinal fluid of RA patients contained increased levels of IL-1β compared to multiple sclerosis (MS) patients and healthy controls (63). Moreover, longitudinal proteomic analyses of CSF samples derived from seven RA patients prior to and during infliximab treatment identified a set of immune-associated markers including complement factor B, which were reduced by TNF blockade (64). Future studies are needed to translate further key aspects of neuroinflammation detected in rodent arthritis models into human diseased conditions.

STRUCTURAL ALTERATIONS AND NEURONAL DYSFUNCTION IN RA

To eventually result in neuropsychiatric symptoms, neuroinflammation is associated with impaired neuronal function. In this regard, several mechanisms have been proposed: alterations in neurotransmitter signaling, dynamic modulation of dendritic spines and neuronal networks, and impaired adult hippocampal neurogenesis.

First, chronic peripheral inflammation was reported to affect glutamatergic and serotonergic signaling. In a rat model of 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced colitis, hippocampal inflammation triggered altered glutamatergic signal transduction, which was reversible upon anti-inflammatory treatment (65). In the pathogenesis of depression, serotonin, a derivative of the essential amino acid tryptophan, plays a key role. Krishnadas et al. observed that serotonin transporter activity in the brainstem assessed by nuclear imaging positively correlated with serum levels of TNF and depressive symptoms (66). In patients with psoriatic arthritis, treatment with the TNF antagonist etanercept for 6–8 weeks significantly reduced serotonin transporter activity, thereby increasing serotonin availability in the synaptic cleft (66). Collectively, chronic peripheral inflammation in RA may induce neurotransmitter dysregulation in the CNS. As neurotransmitter metabolism and recycling is a major homeostatic function of astrocytes, a better understanding of astrocytic modulation during RA-induced neuroinflammation may also provide further insights into the role of different neurotransmitters in arthritis-associated neuropsychiatric symptoms.

Secondly, changes in neuronal structure subsequent to chronic peripheral inflammation may account for neuropsychiatric symptoms. In acute inflammation induced by Poly(I:C), Garré et al. observed increased loss of cortical dendritic spines functionally resulting in learning deficits. Of note, these changes were independent of microglia, but orchestrated by peripheral C-X3-C motif chemokine receptor 1 (Cx3cr1)-expressing monocytes and TNF (23). As TNF was linked to depressive symptoms in AIA (62), it is of great interest to examine dendritic spine dynamics in RA mouse models. Besides remodeling dendritic spines, chronic peripheral inflammation may induce complex structural network rearrangements in the brain. Schrepf et al. demonstrated rewiring of the brain connectome by multimodal brain MRI in RA patients. In particular, the inferior parietal lobe and the medial prefrontal cortex were more strongly involved in several brain networks compared to healthy controls (67).

Adult hippocampal neurogenesis, the generation of new neurons in the dentate gyrus throughout lifetime, is a physiological process contributing to learning, memory, pattern separation and emotions. Adult hippocampal neurogenesis is impaired in models of neurodegenerative diseases (68) and depression (69), but is also affected by local and systemic inflammation (24, 70). It has been investigated in several RA models. Interestingly, in rodent models mimicking the lymphoid subtype of RA, divergent findings were obtained. In AIA, a slight increase in the proliferation of neural progenitor cells and the number of surviving newly generated neurons were described (71, 72). In contrast, Andersson et al. recently showed reduced adult hippocampal neurogenesis and smaller hippocampal volume in mice with CIA (61). Impaired adult hippocampal neurogenesis was mediated by inflammation-induced insulin-like growth factor 1 receptor (IGF1R) signaling. Interestingly, small hippocampal volume in female RA patients correlated with more severe pain and reduced levels of serum insulin-like growth factor 1 (IGF1) (61). Moreover, high IGF1R expression in leukocytes of RA patients significantly correlated with symptoms of anxiety (61). Of note, the study was restricted to female RA patients. It will be interesting to study the role of IGF1/IGF1R in male RA patients, as the role of gender in CNS involvement during RA is hardly investigated. Taken together, the IGF1/IGF1R-axis might therefore serve as a biomarker for some neuropsychiatric symptoms in RA patients. In contrast to lymphoid-based models, a stepwise characterization of adult hippocampal neurogenesis in the myeloid-like hTNFtg model revealed no difference compared to wt controls (58). This is in line with the observed resilience of the hippocampus to neuroinflammation in this model (45, 58).

In summary, chronic peripheral inflammation during arthritis is propagated to the CNS and subsequently causes neuropsychiatric symptoms by affecting neurotransmitter metabolism, dendritic spine and neuronal network dynamics adult hippocampal neurogenesis. It is important to note, that these changes appear to depend on the particular peripheral immunophenotype.

CNS MODULATION OF ARTHRITIS

After highlighting the modulation of the CNS by RA, it is noteworthy that recent studies propose effects of the CNS on the severity and progression of arthritis, which are mainly mediated by the autonomic nervous system. These findings illustrate the reciprocal interaction between CNS pathology and RA and are in line with the clinical observation that depression is frequent in RA, but also predisposes for RA development (73).

To date, different mechanisms of systemic immune modulation by the CNS have been revealed. As a major efferent route mediating immune suppression by the CNS, the vagal nerve controls the production of TNF and other pro-inflammatory cytokines (74). This effect is mediated *via* the splenic nerve, which directly activates β-adrenergic receptors on splenic $CD4^+$ T cells expressing choline acetyltransferase (ChAT). $ChAT^+CD4^+$ T cells in turn suppress cytokine production in other immune cells by cholinergic signaling

via the α7 nicotinic acetylcholine receptor (75, 76). The therapeutic potential of vagal nerve stimulation has been demonstrated in a rat model of CIA (77) and a small group of RA patients, leading to attenuated cytokine production and decreased disease activity scores (78). Moreover, direct stimulation of splenic nerve terminals by ultrasound altered gene expression profiles of B and T cells and alleviated arthritis in the K/BxN serum transfer model, which is mediated by antibodies against glucose-6-phosphate isomerase (79). To date, it is hardly understood, how this modulation of chronic peripheral inflammation *via* the autonomic nervous system is orchestrated by central brain regions. Two recent studies showed that central stimulation of the locus coeruleus and the parietal cortex dampened zymosan-induced arthritis in rats *via* sympathetic adrenergic signaling to affected joints (80, 81). Interestingly, both brain regions are activated by afferent vagal stimulation (80, 81).

Efferent modulation of peripheral inflammation by the CNS raises the question, how neurological diseases influence the risk and severity of arthritis. Lang et al. observed that neurodegeneration in transgenic mice expressing the human tau P301S mutant (P301S mice) was linked to higher induction rates and earlier onset of CIA (44). As the number of $ChAT^+$ T cells in the spleen was not different in P301S mice (44), CNS modulation of arthritis might be independent of the vagus efferent pathway.

Post-stroke immunosuppression is a transient condition of mitigated immune function and increased vulnerability towards infection following cerebral ischemia. This phenomenon was recently linked to the suppression of peripheral natural killer (NK) cells by catecholaminergic and glucocorticoid signaling *via* the sympathetic nervous system and the hypothalamo-pituitary-adrenal axis (82). Intriguingly, post-stroke immunosuppression alleviated K/BxN serum transfer-induced arthritis in mice during early disease stages (83). Collectively, these data suggest that certain CNS regions may on the one hand be a target, but on the other hand also a modulator of chronic peripheral inflammation in the context of RA.

CONCLUSION

Besides joint pathology, patients with RA frequently show highly relevant comorbidities involving the CNS resulting in aggravated therapeutic response and outcome. Neuropsychiatric and neurodegenerative disorders associated with RA are thought to be at least partially linked to neuroinflammation targeting specific brain regions. However, the extent of neuroinflammation and how much it contributes to CNS comorbidities in RA is still unclear. More studies targeting the brain myeloid cell compartment in RA and other peripheral immune diseases like ulcerative colitis would shed more light on the pathogenesis of CNS comorbidities. In this regard, the amelioration by TNF inhibitors like infliximab strongly implies an RA-linked neuroinflammatory response different from MS, which is aggravated or triggered by TNF inhibition (84). The CNS myeloid cell activation pattern in RA was reported to be similar to the disease-associated microglia (DAM) profile observed in neurodegenerative diseases (45, 85), an activation state distinct from the pattern observed in the experimental autoimmune encephalomyelitis model of MS (86). One exciting line of research would be to explore a potential link of complement-dependent synaptic degeneration as postulated in AD (87, 88) in the context of RA.

The heterogeneity in BBB modulation, myeloid cell activation, regional neuroinflammation, and adult hippocampal neurogenesis observed in different RA mouse models will have to be related to the distinct immunophenotypes in RA patients. A major goal is the identification of novel biomarkers defining RA patients at risk of CNS involvement to enable an early interdisciplinary treatment. Vice versa, such biomarkers may help to predict the risk of future arthritis in patients with pre-existing neuropsychiatric diseases, such as depression.

Finally, the bidirectional interaction between chronic peripheral inflammation and the brain will enable innovative treatment approaches for systemic inflammatory, neurological, and psychiatric diseases.

AUTHOR CONTRIBUTIONS

PS and TR drafted the manuscript. PS designed the figure. PS, AH, JS, and JW discussed and revised the manuscript. All authors contributed to the article and approved the submitted version.

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Interleukin 10 Restores Lipopolysaccharide-Induced Alterations in Synaptic Plasticity Probed by Repetitive Magnetic Stimulation

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Systemic inflammation is associated with alterations in complex brain functions such as learning and memory. However, diagnostic approaches to functionally assess and quantify inflammation-associated alterations in synaptic plasticity are not well-established. In previous work, we demonstrated that bacterial lipopolysaccharide (LPS)-induced systemic inflammation alters the ability of hippocampal neurons to express synaptic plasticity, i.e., the long-term potentiation (LTP) of excitatory neurotransmission. Here, we tested whether synaptic plasticity induced by repetitive magnetic stimulation (rMS), a non-invasive brain stimulation technique used in clinical practice, is affected by LPS-induced inflammation. Specifically, we explored brain tissue cultures to learn more about the direct effects of LPS on neural tissue, and we tested for the plasticity-restoring effects of the anti-inflammatory cytokine interleukin 10 (IL10). As shown previously, 10 Hz repetitive magnetic stimulation (rMS) of organotypic entorhino-hippocampal tissue cultures induced a robust increase in excitatory neurotransmission onto CA1 pyramidal neurons. Furthermore, LPS-treated tissue cultures did not express rMS-induced synaptic plasticity. Live-cell microscopy in tissue cultures prepared from a novel transgenic reporter mouse line [*C57BL/6-Tg(TNFa-eGFP)*] confirms that *ex vivo* LPS administration triggers microglial tumor necrosis factor alpha (TNFα) expression, which is ameliorated in the presence of IL10. Consistent with this observation, IL10 hampers the LPS-induced increase in TNFα, IL6, IL1β, and IFNγ and restores the ability of neurons to express rMS-induced synaptic plasticity in the presence of LPS. These findings establish

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organotypic tissue cultures as a suitable model for studying inflammation-induced alterations in synaptic plasticity, thus providing a biological basis for the diagnostic use of transcranial magnetic stimulation in the context of brain inflammation.

Keywords: synaptic plasticity, neuroinflammation, non-invasive brain stimulation, transcranial magnetic stimulation, TNFα-reporter mouse, interleukin 10

INTRODUCTION

Alterations in cognitive function and behavior are often observed in the context of systemic inflammation and/or infection of the central nervous system (1–3). Several immune mediators that affect the ability of neurons to express plasticity have been identified (4, 5). This is of considerable relevance in the context of neurological and psychiatric diseases associated with increased levels of pro-inflammatory cytokines in the brain (6–9). However, diagnostic approaches to functionally assess and quantify inflammation-associated alterations in neuronal plasticity and to monitor the effects of (plasticity-restoring) therapeutic interventions as yet remain underdeveloped.

Transcranial magnetic stimulation (TMS) is a promising clinical tool in this context. Based on the principle of electromagnetic induction, TMS allows for the non-invasive stimulation of cortical brain regions through the intact skin and skull (10). The effects of single TMS pulses over the primary motor cortex can be quantified by recording motor evoked potentials in the target muscle (11). Consecutive trains of TMS pulses (repetitive TMS, rTMS) over the motor cortex promote enduring changes of cortical excitability, i.e., neural plasticity (12). For example, high-frequency rTMS (≥ 5 Hz) typically increases cortico-spinal excitability, measured as an increase in the amplitude of motor-evoked potentials (13–15). Pharmacological approaches and analogies to basic research findings provide evidence to suggest that high-frequency rTMS asserts its effects via "long-term potentiation (LTP)-like" plasticity (16, 17). Previous studies employing animal models of rTMS (both in vitro and in vivo) have revealed that rTMS is capable of inducing long-lasting changes in glutamatergic neurotransmission that are consistent with the LTP of excitatory neurotransmission (18–22). Since rTMS induces plasticity phenomena, in the present study, we hypothesize that this form of plasticity will be affected by neural inflammation.

In a previous study we reported that bacterial lipopolysaccharide (LPS)-induced systemic inflammation affects the ability of neurons to express LTP at the Schaffer collateral-CA1 synapses in the hippocampus (23). While it is suspected that LPS can act directly on neuronal tissue to induce inflammation and alterations in synaptic plasticity, direct evidence for this remains scarce. Furthermore, the major source of the pro-inflammatory cytokine tumor necrosis factor alpha (TNFα), which has been linked to synaptic plasticity modulation (24–27) is not well-characterized, while recent data suggest a distinct role of microglia derived TNFα in the regulation of LTP (28). Therefore, in the present study, we employed *in vitro* LPS exposure of entorhino-hippocampal tissue cultures prepared from a novel TNFα-reporter mouse strain that expresses the enhanced green fluorescent protein (eGFP) under the control of the *Tnfa* promoter [*C57BL/6-Tg (TNFa-eGFP)*] to monitor LPS-induced neural inflammation. Following our previous work on the cellular and molecular effects of repetitive magnetic stimulation (rMS) (19, 21, 29) and based on our recent findings of TNFα-mediated changes in synaptic plasticity (30), we tested whether excitatory synaptic plasticity induced by 10 Hz rMS is affected by *in vitro* LPS-induced inflammation, and whether the anti-inflammatory cytokine interleukin 10 (IL10) restores rMS-induced synaptic plasticity.

MATERIALS AND METHODS

Ethics Statement

Mice were maintained in a 12 h light/dark cycle with food and water available *ad libitum*. Every effort was made to minimize distress and pain of animals. All experimental procedures were performed according to the national animal welfare legislation and approved by the animal welfare committee (V54-19c20/15-F143/37), the local animal welfare officers (Universities of Frankfurt, Mainz and Freiburg) and/or the institutional animal care and use committee (Sheba Medical Center).

C57BL/6-Tg(TNFa-eGFP) Reporter Mouse Line

To create a genetic construct that allows for the visualization of TNFα expression through simultaneous eGFP expression, the bacterial artificial chromosome (BAC) RP23-115O3 (GenBank: CR974444.18, BACPAC resources center at the children's hospital Oakland research institute [CHORI]) was used as follows: Rabbit β-globin intron (Ac. No.: J00659; nucleotides: 907-1550) was amplified by the use of polymerase chain reaction (PCR) and cloned into pBluescript KS^+ (Stratagene) via ClaI-EcoRI sites. The eGFP construct/sequence fused to the SV40 early mRNA polyadenylation (PA) signal was generated by applying standard PCR technique, using pEGFP-N1 (clontech) as a template and appropriate primers to delete the NotI site and cloned downstream to the Rabbit β-globin intron via EcoRI-SalI sites (all primers are given in the supplementary information, **Table S1**). The murine *Tnfa* promoter sequence including the first 167 nucleotides of exon 1 (Ac. No.: Y00467.1; nucleotides: 3120-4526) was inserted upstream to the rabbit β-globin intron via NotI-HindIII sites. A PGK-EM-neo cassette flanked by two full FRT sites was amplified using appropriate primers and the YTT vector B (Ac. No.: AY028413.1; nucleotides: 410-2310) as a

template using standard PCR protocol and cloned as a SalI-NheI fragment downstream to the SV40 PA sequence. Finally, the DNA coding sequence immediately after the *Tnfa*'s PA signal (BAC RP23-115O3; nucleotides: 135366-134412) was obtained by PCR amplification and cloned downstream to the neo cassette via NheI-KpnI sites. The BstBI-KpnI fragments containing the coding sequences of the *Tnfa* promoter, the eGFP construct, the neo cassette and the *Tnfa* downstream region were used to replace the *Tnfa* coding sequence (nucleotides: 137835 to 135366) at BAC RP23-115O3 by the use of Red ET homologous recombination methods (31). Positive BAC clones were analyzed by PCR using the upstream (U1-2a) and downstream (D1-2a) primers. BACs that were cloned correctly were further evaluated by southern blotting using the 5'- and the 3'-probes. Finally, the 5'- and the 3'-recombination junctions were sequenced to verify the correct integration sites of the reporter construct into the *Tnfa* locus. The recombinant eGFP reporter BAC was linearized using the restriction enzyme BsiWI (Thermo Scientific) and purified using a CL4b sepharose column (Pharmacia). Eluted DNA was diluted to 1 µg/ml in an injection buffer (10 mM Tris, 0.1 mM EDTA, 100 mM NaCl, pH 7.5 with HCl) and injected into pronuclei oocytes (C57BL/6J-DBA hybrid). Transgenicity of the founders was verified by standard PCR methods using primer pairs corresponding to the Rabbit β-globin intron and eGFP sequence. The eGFP reporter efficiency was tested *in vitro* and *in vivo* and three founder strains were selected for additional experiments. Finally, to avoid disturbance of the neo gene, transgenic mice were bred with *FLP* mice (Jackson laboratory) and the neo-deleted offspring was backcrossed to *C57BL/6J* mice. Transgenic progeny was born at a normal Mendelian ratio and showed no signs of developmental abnormality or inflammatory insult.

Preparation of Tissue Cultures

Organotypic entorhino-hippocampal tissue cultures were prepared at postnatal day 3–5 from *C57BL/6J* (Charles River) and heterozygous *C57BL/6-Tg(TNFa-eGFP)* reporter mice of either sex as previously described (32). Tissue cultures were grown on porous inserts with 0.4 µm pore size (30 mm, hydrophilic PTFE, Millipore PICM0RG50). Culturing medium contained 50% (v/v) MEM, 25% (v/v) basal medium eagle, 25% (v/v) heat-inactivated normal horse serum (NHS), 25 mM HEPES buffer solution, 0.65% (w/v) glucose, 0.15% (w/v) bicarbonate, 0.1 mg/ml streptomycin, 100 U/ml penicillin and 2 mM glutamax. The pH was adjusted to 7.30 and tissue cultures were incubated for at least 18 days at 35°C in a humidified atmosphere with 5% CO_2. Culturing medium was replaced by fresh medium three times per week.

Pharmacology

Tissue cultures (≥ 18 days in vitro) were treated with lipopolysaccharide (1 µg/ml; LPS from Escherichia coli O111: B4, Sigma #L4391) for 3 days [c.f., (33)], which induced a robust increase in *Tnfa*-mRNA levels (**Figure 3**) that is comparable to what has been reported *in vivo* (23). In some experiments, interleukin 10 (10 ng/ml; mouse recombinant, R&D Systems #417-ML) was added to the culturing medium. Age- and time-matched control cultures were treated with vehicle-only in the same way.

Live-Cell Imaging

Live-cell imaging of heterozygous *C57BL/6-Tg(TNFa-eGFP)* cultures was performed at a Zeiss LSM800 microscope equipped with a 10x water-immersion objective (NA 0.3; Carl Zeiss). Filter membranes with 3 to 6 cultures were placed in a 35 mm Petri Dish containing pre-oxygenated imaging solution consisting of 50% (v/v) MEM, 25% (v/v) basal medium eagle, 50 mM HEPES buffer solution (25% v/v), 0.65% (w/v) glucose, 0.15% (w/v) bicarbonate, 0.1 mg/ml streptomycin, 100 U/ml penicillin, 2 mM glutamax, and 0.1 mM trolox. The cultures were kept at 35°C during the imaging procedure. Laser intensity and detector gain were initially set to keep the fluorescent signal in a dynamic range throughout the experiment and were kept constant in all experiments. Of each culture a z-stack was recorded before treatment (day 0) with $\Delta z = 6.3$ µm at ideal Nyquist rate and an optical zoom of 0.5. After imaging, cultures were returned to the incubator and treatment was started. The imaging procedure was repeated after 3 days of treatment (day 3) following the same experimental protocol with the exact same imaging parameters. Confocal image stacks were stored as .czi files.

Immunostaining and Imaging

Tissue cultures were fixed in a solution of 4% (w/v) paraformaldehyde (PFA) in PBS (0.1 M, pH 7.4) and 4% (w/v) sucrose for 1 h, followed by 2% PFA and 30% sucrose in PBS (0.1 M, pH 7.4) overnight. Cryostat sections (30 µm; 3050CM S, Leica) of fixed slice cultures were prepared and stained with antibodies against Iba1 (rabbit, 1:1,000; Fujifilm Wako #019-19741) and GFAP (mouse, 1:1,000; Sigma-Aldrich #G3893). Slices were incubated in appropriate Alexa Fluor® dye-conjugated secondary antibodies (donkey, 1:1,000; invitrogen #A-21206). For post-hoc staining, slices were incubated with Alexa Fluor® 488-conjugated streptavidin (1:1,000; invitrogen #S32354). TO-PRO®-3 (1:5,000 in PBS for 10 min; invitrogen #T3605) nuclear stain or DAPI nuclear stain (1:5,000 in PBS for 20 min; Thermo Scientific #62248) was used to visualize cytoarchitecture. Sections were washed, transferred on glass slides and mounted for visualization with anti-fading mounting medium (Agilent #S302380-2). Confocal images were acquired using a Nikon Eclipse C1si laser-scanning microscope equipped with a 20x (NA 0.75; Nikon) and a 40x oil-immersion objective (NA 1.3; Nikon).

Propidium Iodide Staining

Tissue cultures were incubated with propidium iodide (PI, 5 µg/ml; invitrogen #P3566) for 2 h, washed in PBS (0.1 M, pH 7.4) and fixed as described above. Cultures treated for 4 h with NMDA (50 µg/ml; Tocris #0114) and cultures incubated for 2 h with PI after fixation (referred to as 'post-fixation') served as positive controls in these experiments. Cell nuclei were stained with TO-PRO®-3 (1:5,000 in PBS for 10 min; invitrogen #T3605). Cultures were mounted on microscope slides and confocal images were acquired as described above.

Quantitative Reverse Transcription PCR (RT-qPCR)

Living tissue cultures were transferred to RLT-buffer (QIAGEN) without any pre-experimental processing or storage and the tissue was immediately homogenized using micropistills. RNA from individual cultures was isolated according to the manufacturer's instructions (RNeasy Plus Micro Kit, QIAGEN #74034). Each sample represents a biological replicate. RNA quantification was performed using a Nanodrop 2000 by assessing the optical density of the RNA sample (dissolved in water). Purified RNA was consecutively reverse transcribed (RevertAid RT Kit, Thermo Scientific #K1691) according to the manufacturer's instruction and the cDNA was diluted in water to a final concentration of 4 ng/µl. RNA/cDNA-integrity was assessed by RT-qPCR of two different transcripts in different locations (Mm01352359_m1; Mm01352366_m1) of the *Sdha* gene in non-pre amplified cDNA. Only cDNA with $\Delta Cq < 0.3$ (mean Cq-difference: 0.113 ± 0.01) between the two probes was further analyzed.

For mRNA correlations with the cytokine detection assay, cDNA preamplification was performed according to the manufacturer's instruction (TaqMan™ PreAmp Master Mix, Applied Biosystems #4391128) with 25 ng cDNA input and 14 preamplification cycles.

RT-qPCR was performed using a C1000 Touch Thermal Cycler (BIO-RAD) and the CFX 384 Real-Time PCR system (BIO-RAD). 18 ng target cDNA (without preamplification) or the recommended amount of preamplified cDNA (according to manufacturer's instructions, TaqMan™ PreAmp Master Mix, Applied Biosystems #4391128) diluted in TaqMan Gene Expression Master Mix (Applied Biosystems #4369016) was amplified using standard TaqMan gene expression assays (Applied Biosystems; Assay-IDs: *Gapdh*: Mm99999915_g1; *Tnf*: Mm00443258_m1; *Il1b*: Mm00434228_m1; *Il6*: Mm00446190_m1; *Il10*: Mm01288386_m1; *Ifng*: Mm01168134_m1). A standard RT-qPCR protocol was used: 1 cycle of 50°C for 2 min, 1 cycle of 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min. Sample amplification was performed in three technical replicates. No amplification was detected in non-template controls. Amplification curves were excluded from further analysis if efficiency values were less than 90 or exceeded 110 according to automated calculation by the Bio-Rad CFX Maestro software package. Data were exported and stored on a computer as .pcrd-files.

Cytokine Detection Assay

For cytokine detection, entorhino-hippocampal tissue cultures were prepared as described above. After preparation, slices were placed on polycarbonate membranes for 24-well plates (two slices per membrane; 0.4 µm pore size, Thermo Scientific #140620). Cultures were allowed to mature for at least 18 days before experimental assessment. Incubation medium from vehicle-only and LPS-treated tissue cultures was collected, frozen immediately in liquid nitrogen, and stored at -80°C. For detection of TNFα, IFNγ, IL1β, IL6, and IL10 a V-Plex Proinflammatory Panel 1 (mouse) Kit Plus (Mesoscale Discovery #K15048G) was used. Cultures were incubated for three days with vehicle-only, LPS or LPS + IL10 in a 24-well plate as described above, and the incubation medium was collected consecutively. To match gene expression with protein content in the incubation medium, cultures were harvested and processed for RT-qPCR analysis. The collected incubation medium was diluted 1:1 in diluent provided with the kit. Protein detection was performed according to manufacturer's instructions. A pre-coated plate with capture antibodies on defined spots was incubated with the diluted samples for 2 h. After washing, samples were incubated for 2 h with a solution containing electrochemiluminescent MSD SULFO-TAG detection antibodies (Mesoscale Discovery; Antibodies: Anti-ms IFNγ Antibody #D22QO, Anti-ms IL1β Antibody #D22QP, Anti-ms IL6 Antibody #D22QX, Anti-ms IL10 Antibody, #D22QU, Anti-ms TNFα Antibody #D22QW). After washing, samples were measured with a MESO QuickPlex SQ 120 instrument (Mesoscale Discovery). The respective protein concentrations were determined using the MSD DISCOVERY WORKBENCH software (Mesoscale Discovery).

Repetitive Magnetic Stimulation

Filter membranes containing tissue cultures were transferred to a 35 mm Petri Dish filled with preheated (35°C) standard extracellular solution containing (in mM) 129 NaCl, 4 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 4.2 glucose, 10 HEPES, 0.1 mg/ml streptomycin, 100 U/ml penicillin, pH 7.4 with KOH, 380–390 mOsm with sucrose. Cultures were stimulated using a Magstim Rapid^2 stimulator (Magstim Company, UK) that is connected to a Double AirFilm® Coil (coil parameters according to manufacturer's description: average inductance = 12 µH; pulse rise time approximately 80 µs; pulse duration = 0.5 ms, biphasic; Magstim Company, UK). Cultures were oriented (~1 cm beneath the center of the coil) in a way that the induced electric field was parallel to the dendritic tree of CA1 pyramidal neurons. The stimulation protocol consists of 900 pulses at 10 Hz (at 50% of maximum stimulator output). After stimulation, cultures were immediately transferred into culturing medium and kept in the incubator for at least 2 h before experimental assessment. Age- and time-matched control cultures were not stimulated but otherwise treated in the same way.

Whole-Cell Patch-Clamp Recordings

Whole-cell patch-clamp recordings from CA1 pyramidal cells of tissue cultures were carried out at 35°C as described previously (3–7 cells per culture). The bath solution contained (in mM) 126 NaCl, 2.5 KCl, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 2 $CaCl_2$, 2 $MgCl_2$, 10 glucose. Patch pipettes contained (in mM) 126 K-gluconate, 10 HEPES, 4 KCl, 4 ATP-Mg, 0.3 GTP-Na_2, 10 PO-Creatine, 0.3% (w/v) biocytin (pH 7.25 with KOH, 290 mOsm with sucrose), having a tip resistance of 4–6 MΩ. In some experiments Alexa 488 (10 µM; invitrogen #A10436) was added to the internal solution to visualize cellular morphology during electrophysiological assessment. Spontaneous excitatory postsynaptic currents (sEPSCs) were recorded at a holding potential of -60 mV. Series resistance was monitored before and after each recording and recordings were discarded if the

series resistance reached ≥30 MΩ. For mEPSC recordings, D-APV (10 μM; Abcam #ab120003) and TTX (0.5 μM; Biotrend #18660-81-6) were added to the external solution. For recording of intrinsic cellular properties in current-clamp mode, pipette capacitance of 2.0 pF was corrected and series resistance was compensated using the automated bridge balance tool of the multiclamp commander. IV-curves were generated by injecting 1 s square pulse currents starting at -100 pA and increasing in 10 pA steps until +500 pA current injection was reached (sweep duration: 2 s).

Quantification and Statistics

Since cultures were grown individually, data obtained from one tissue culture were considered as one independent experiment. Biological replicates were identified according to previously established standards (34), and were displayed as individual data points in the figures.

RT-qPCR data were analyzed with the Bio-Rad CFX Maestro 1.0 software package using the ΔΔCq method with *Gapdh* as reference gene. Values were normalized to the mean value of the respective vehicle-treated control group.

Colocalization analysis of GFP and Iba1/GFAP was assessed using the "coloc2" plugin of Fiji image processing package [available at https://fiji.sc/ (35)] and Pearson's correlation coefficients (also referred to as Pearson's r) were obtained. As a control, Pearson's r values were calculated from the same set of images in which the Iba1 channel was rotated by 180°.

Mesoscale cytokine detection assay was analyzed using the MSD DISCOVERY WORKBENCH 4.0 platform. mRNA/protein correlations were analyzed using Pearson's correlation coefficients (r).

For PI stainings, fluorescence intensity was analyzed in CA1, *stratum pyramidale*. Values were normalized to the mean fluorescence intensity in the vehicle-only treated group.

Confocal image stacks of heterozygous *C57BL/6-Tg(TNFa-eGFP)* cultures were processed and analyzed using the Fiji image processing package. Of each stack a maximum intensity projection followed by background subtraction (rolling ball radius: 50 px) was performed. Culture area was manually defined as a Region of Interest (ROI) and the mean fluorescence intensity of the ROI was calculated. Mean fluorescence intensity of the culture area was normalized to the respective values at day 0 in the same culture.

Single cell recordings were analyzed off-line using Clampfit 11 of the pClamp11 software package (Molecular Devices). sEPSC properties were analyzed using the automated template search tool for event detection. Intrinsic cellular properties were analyzed using Clampfit 11. Input resistance was calculated for the injection of -100 pA current at a time frame of 200 ms with maximum distance to the Sag-current.

Statistical comparisons were performed using non-parametric tests (Mann-Whitney test, Kruskal-Wallis test followed by Dunn's multiple comparisons). In culture comparison in time-lapse imaging experiments was performed using the Wilcoxon matched-pairs signed rank test. Statistical analysis of cytokine detection assay depicted in **Figure 5** was performed using a one-way ANOVA test followed by Holm Sidak's multiple comparison test. p values smaller 0.05 were considered a significant difference. In the text and figures, values represent mean ± standard error of the mean (s.e.m.). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and not significant differences are indicated by "ns". U values are reported in the figure legends for significant results only.

Digital Illustrations

Figures were prepared using the Photoshop graphics software (Adobe, San Jose, CA, USA). Image brightness and contrast were adjusted.

RESULTS

rMS-Induced Synaptic Plasticity Is Impaired in LPS-Treated Tissue Cultures

The effect of 10 Hz rMS on excitatory synaptic strength was tested in entorhino-hippocampal tissue cultures (≥ 18 days in vitro; **Figures 1A, B**) prepared from *C57BL/6* mice of both sexes. Tissue cultures were treated with 1 μg/ml of LPS or vehicle-only for three days before the experimental assessment, and AMPA receptor-mediated miniature excitatory postsynaptic currents (mEPSCs) were recorded from individual CA1 pyramidal neurons in whole-cell configuration after the treatment period (**Figures 1C, D**). Postsynaptic strength was reflected in the amplitude of inward current responses, which are evoked by the stochastic release of glutamate from presynaptic terminals (**Figure 1C**). Consistent with our previous work (19, 21), increased mEPSC amplitudes were observed 2–4 h following 10 Hz rMS in vehicle-only tissue cultures. Conversely, CA1 neurons of tissue cultures that had been treated with LPS (1 μg/ml, 3 days) prior to magnetic stimulation failed to potentiate their excitatory synapses following rMS (**Figure 1D**). Importantly, the baseline mEPSC amplitudes of vehicle-only and LPS-treated cultures were not significantly different (**Figure 1D**). These results demonstrate that LPS administered directly to brain tissue impairs the ability of neurons to express rMS-induced synaptic plasticity without affecting excitatory synaptic strength.

To corroborate these findings, LTP experiments utilizing local electric stimulation of Schaffer collateral-CA1 synapses were carried out in acute slice preparations that had been treated with LPS (1 μg/ml) prior to LTP induction (1 s, 100 Hz; **Figure S1**). The ability of CA1 pyramidal neurons to express LTP was altered in these experiments, while no changes in baseline synaptic transmission were observed following the application of LPS (at 15 min for ~ 6 h, i.e., prior to LTP induction in the LPS-treated group; **Figure S1**). These findings agree with our previous *in vivo* work (23), thus validating the use of *in vitro* LPS exposure as a method to study neuroinflammation-associated alterations in synaptic plasticity probed by 10 Hz repetitive magnetic stimulation of organotypic tissue cultures.

Together, these experiments demonstrate that both rMS-induced synaptic plasticity and classic electric tetanus-induced LTP are altered by direct exposure to LPS.

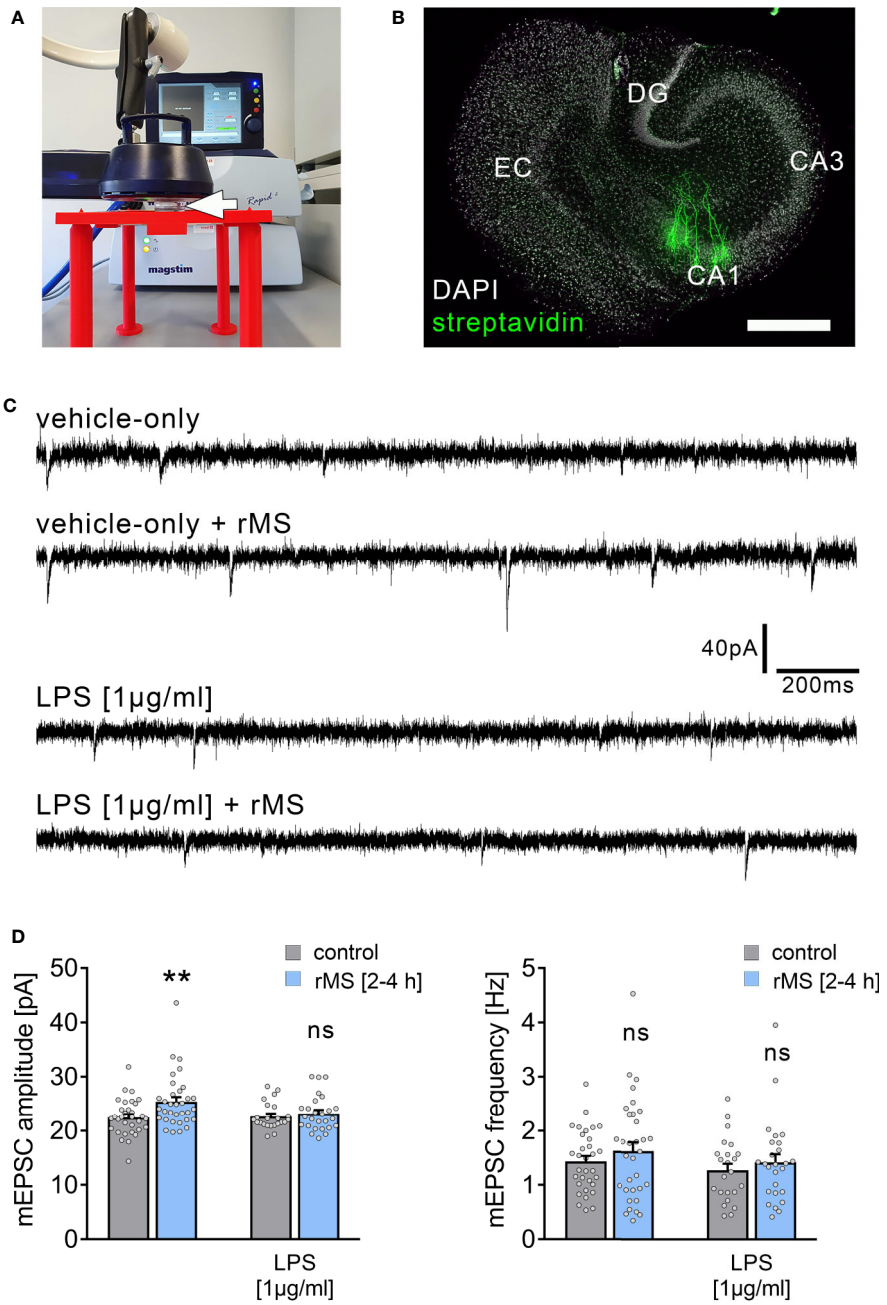

FIGURE 1 | Synaptic plasticity induced by repetitive magnetic stimulation is impaired in entorhino-hippocampal tissue cultures exposed to Lipopolysaccharide. **(A)** Repetitive magnetic stimulation of organotypic entorhino-hippocampal tissue cultures was performed using a Magstim Rapid² stimulator equipped with an AirFilm® stimulation coil. Cultures were placed in a Petri dish below the center of the coil (white arrow) on a custom-designed 3D-printed stage. Distance to coil and orientation of tissue cultures within the electromagnetic field was kept constant in all experiments. A 10 Hz rMS protocol consisting 900 pulses was used to probe excitatory synaptic plasticity. **(B)** Visualization of CA1 pyramidal neurons using Alexa Fluor 488-labeled streptavidin. Scale bar: 500 µm. EC, entorhinal cortex; DG, dentate gyrus. **(C, D)** Representative traces and group data of miniature excitatory postsynaptic currents (mEPSC) recorded from individual CA1 pyramidal neurons 2–4 h after stimulation in the respective groups. Tissue cultures that had been treated with LPS (1 µg/ml, 3 days) prior to magnetic stimulation fail to potentiate their excitatory synapses (vehicle-only: $n_{control}$ = 32 cells in 6 cultures, $n_{rMS\ [2\text{-}4h]}$ = 33 cells in 6 cultures; Mann-Whitney test, U = 330; LPS: $n_{control}$ = 23 cells in 4 cultures, $n_{rMS\ [2\text{-}4h]}$ = 26 cells in 4 cultures; Mann-Whitney test; one recording excluded in the rMS/vehicle-only group with 65.25 pA/3.17 Hz). Individual data points are indicated by gray dots. Values represent mean ± s.e.m. (** $p < 0.01$; ns, not significant difference). U values are provided for significant results only.

LPS-Induced Neural Toxicity Does Not Account for Alterations in rMS-Induced Synaptic Plasticity

Propidium iodide (PI) stainings were used to assess cell viability in our experimental setting. PI is a cell membrane impermeant fluorescent molecule that binds to DNA. Hence, PI can be used as a marker for membrane integrity when applied to living tissue (36).

To test for LPS-induced effects on cell viability in organotypic tissue cultures, another set of cultures was treated with LPS (1 µg/ml, 3 days) and vehicle-only. Formaldehyde fixation, which compromises membrane integrity, and short-term NMDA treatment (50 µM, 4 h) served as positive controls in this series of experiments. **Figure 2** shows that the PI signal was comparably low in the control and LPS-treated groups, while a significant increase in PI signal was evident in the NMDA-treated group, and almost all nuclei were PI-positive following fixation (**Figures 2A–C**).

Considering the clinical relevance of non-invasive magnetic stimulation, i.e., TMS, we also tested for the effects of 10 Hz rMS. No apparent negative effects on cell viability (2–4 h after stimulation) were detected in the vehicle-only controls, which exhibited rMS-induced synaptic plasticity, or in the LPS-treated cultures, in which no increase in excitatory synaptic strength following 10 Hz rMS was evident (**Figure 2B**). According to the above results (**Figure 2D**), we conclude that neither LPS nor 10 Hz rMS significantly affect cell viability. This finding corroborates previous work in which no negative or even neuroprotective effects of rTMS have been reported (37–39).

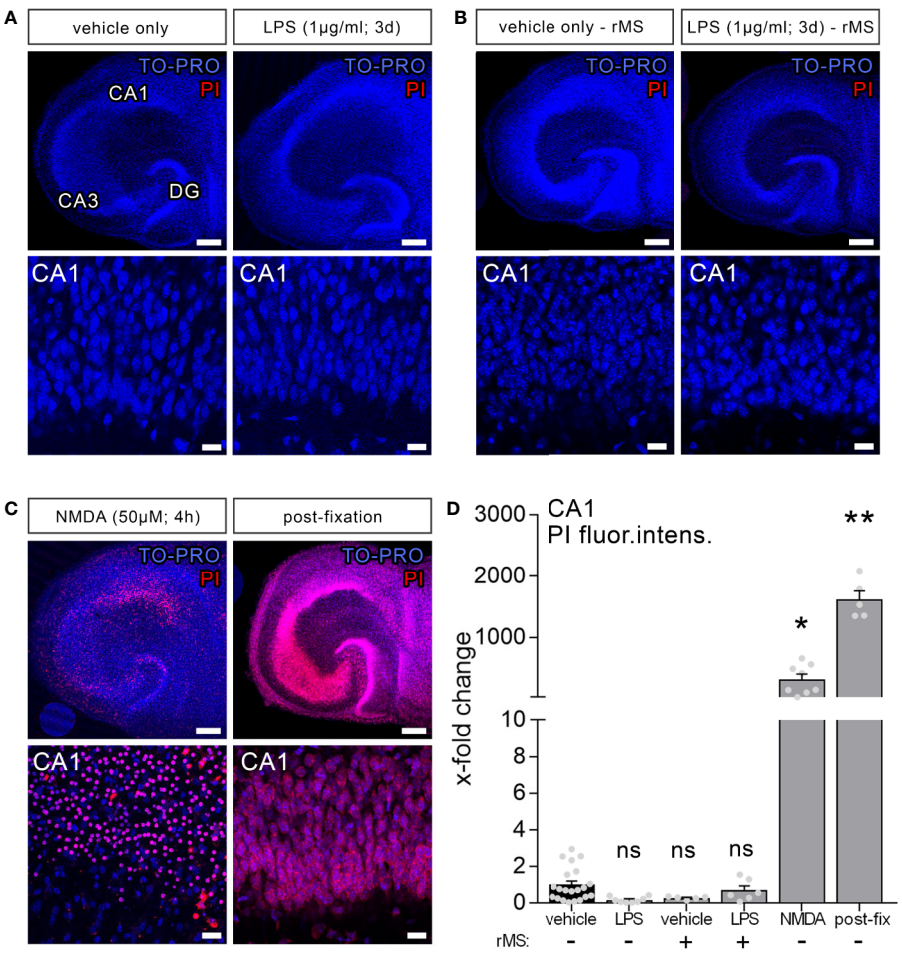

FIGURE 2 | Lipopolysaccharide and/or repetitive magnetic stimulation do not affect cell viability. **(A–C)** Representative examples of propidium iodide staining (PI, red) in the various experimental conditions (TO-PRO nuclear stain, blue). **(A)** No effect on membrane integrity is observed following *in vitro* LPS exposure (1 µg/ml, 3 days) as compared to age- and time-matched vehicle-only treated controls. **(B)** No signs of cell death are detected 2–4 h after rMS, both in vehicle-only and LPS-treated tissue cultures. **(C)** NMDA treatment (50 µM, 4 h) and formalin fixation served as positive controls in these experiments. Scale bars: row at the top 200 µm and row at the bottom 20 µm. **(D)** Summary graph and combined analysis of changes in propidium iodide fluorescence intensity in area CA1 under the indicated experimental conditions, respectively ($n_{vehicle\text{-}only}$ = 21 cultures, n_{LPS} = 8 cultures, n_{rMS} = 5 cultures, $n_{LPS+rMS}$ = 6 cultures, n_{NMDA} = 8 cultures, $n_{post\text{-}fix}$ = 5 cultures; Kruskal-Wallis test followed by Dunn's multiple comparisons). Individual data points are indicated by gray dots. Values represent mean ± s.e.m. (* $p < 0.05$, ** $p < 0.01$; ns, not significant difference).

LPS Triggers Microglial TNFα Expression in Organotypic Tissue Cultures

To further characterize the effects of LPS in our experimental setting, we used entorhino-hippocampal tissue cultures prepared from a novel transgenic reporter mouse line [*C57BL/6-Tg(TNFa-eGFP)*], which expresses eGFP under the control of the *Tnfa* promoter (**Figures 3A, B**). Cultures prepared from these mice were treated with LPS (1 µg/ml, 3 days) or vehicle-only. Baseline fluorescence intensity was measured prior to treatment (day 0) and at the end of the treatment period (day 3) using live-cell microscopy. A significant increase in the eGFP fluorescence was detected in the LPS-treated group as compared to the vehicle-

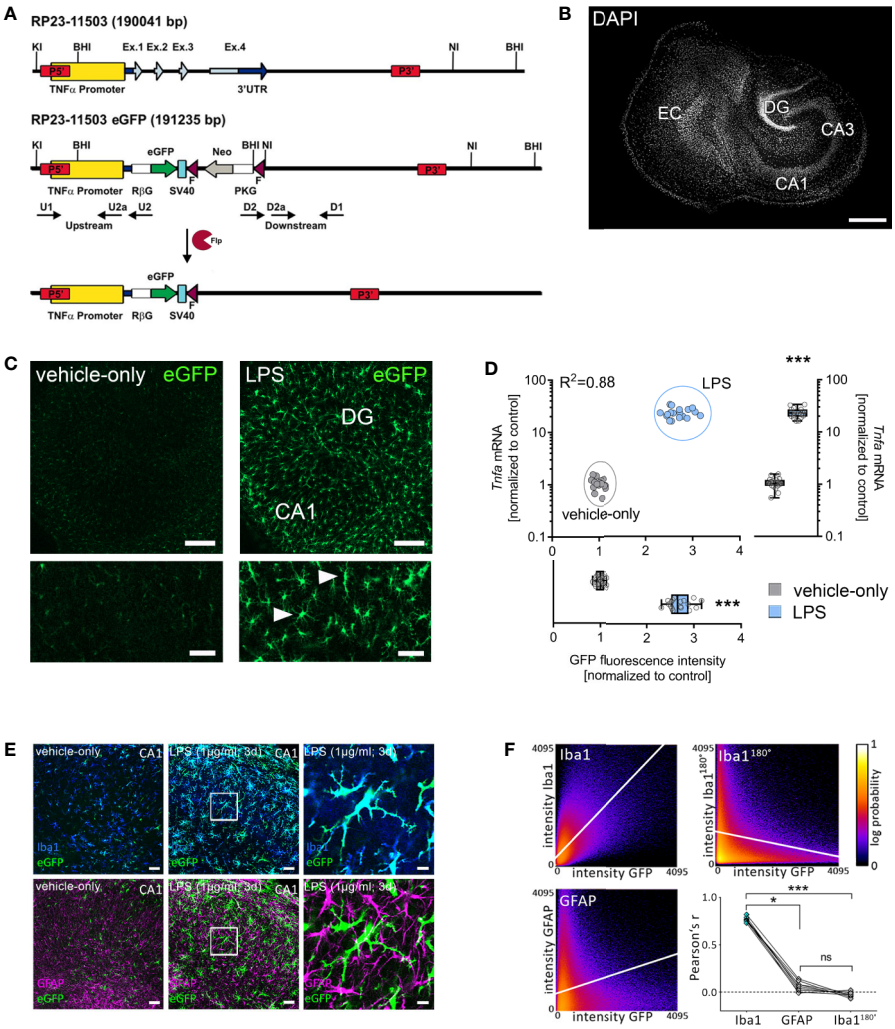

FIGURE 3 | Microglia are a major source of tumor necrosis factor α (TNFα) in tissue cultures exposed to Lipopolysaccharide. **(A, B)** Schematic representation of the *Tg(TNFa-eGFP)* reporter transgenic construct. The *Tnfa* locus of BAC RP23-115O3 containing the *Tnfa* promoter as well as the four exons (Ex. 1 to 4) are shown in the upper part. In the recombinant eGFP BAC (middle part), the *Tnfa* coding sequence and the 3' untranslated region (3'UTR) were replaced by eGFP and neomycin (Neo) coding cassettes. After flippase (Flp) mediated intra-chromosomal recombination, the neo cassette is excised, leaving the Rabbit β-globin intron (RβG)-eGFP fusion gene under the control of the *Tnfa* promoter, as illustrated in the lower part. SV40, SV40 early mRNA polyadenylation; F, flippase recognition target; PKG, phosphoglycerate kinase. A tissue culture stained with DAPI nuclear staining is shown to visualize cytoarchitecture. Scale bar: 400 µm. **(C)** Examples of tissue cultures prepared from *C57BL/6-Tg(TNFa-eGFP)* mice after 3 days (3 d) of treatment with 1 µg/ml LPS (right side) and vehicle-only (left side). A considerable increase in the eGFP signal is observed in the LPS-treated group (smaller sections with higher magnification at the bottom). Scale bars: row at the top 200 µm and row at the bottom 75 µm. **(D)** Quantification of the eGFP signal and the corresponding *Tnfa*-mRNA content of individual cultures in vehicle-only (gray dots) and LPS-treated (blue dots) *C57BL/6-Tg(TNFa-eGFP)* organotypic tissue cultures (n = 18 cultures per group and experimental condition; Mann-Whitney test, $U_{GFP\text{-}intensity} = 0$, $U_{Tnfa\text{-}mRNA} = 0$; Boxplot diagrams indicate the level of significance and distribution of data points within the groups; R^2 was calculated by linear regression analysis). **(E)** Examples of *C57BL/6-Tg(TNFa-eGFP)* tissue cultures treated with vehicle-only or LPS stained for the astrocytic marker GFAP and the microglial marker Iba1. Scale bars: left and middle row 50 µm and right row 10 µm. **(F)** Quantitative assessment of colocalization shows colocalization between the GFP- and the Iba1-signal, but not the GFP- and the GFAP-signal. The same set of images in which the Iba1-signal is rotated 180° served as a control for this analysis (n = 9 cultures per group; Kruskal-Wallis test followed by Dunn's multiple comparisons). Individual data points are indicated by single dots. Values represent mean ± s.e.m. (* $p < 0.05$, *** $p < 0.001$; ns, not significant difference).

only controls, which did not show significant changes in eGFP expression (**Figures 3C, D**). The validity of this experimental approach was further supported by correlated changes between eGFP fluorescence and RT-qPCR analysis for *Tnfa*-mRNA in the same set of tissue cultures (**Figure 3D**).

The majority of eGFP-expressing cells were Iba1 and not GFAP positive as shown by immunostaining of tissue cultures (**Figures 3E, F**). Careful examination of Iba1 and GFAP negative areas in CA1 *stratum pyramidale*, i.e., the layer in which the cell bodies of the recorded CA1 neurons are found, revealed no evidence of neuronal TNFα expression. Although these experiments did not exclude astrocytic and/or neuronal sources of TNFα, our data demonstrate that microglia are a major source of TNFα during LPS-induced neuroinflammation. This observation is in line with previous work that has demonstrated the presence of toll-like receptor 4 (TLR4) on microglia, i.e., the main receptor targeted by LPS (40, 41).

IL10 Attenuates LPS-Induced TNFα Expression in Tissue Cultures Prepared From TNFα-Reporter Mice

Next, we tested for the effects of the anti-inflammatory cytokine IL10 in LPS-treated tissue cultures (**Figure 4**) (42, 43). Tissue cultures prepared from TNFα-reporter mice were again exposed to LPS (1 µg/ml, 3 days), and changes in eGFP fluorescence were monitored using live-cell microscopy (**Figure 4A**). While a robust increase in eGFP fluorescence was detected in the LPS-treated cultures, IL10 co-incubation (10 ng/ml, 3 days) along with LPS attenuated the increase in eGFP fluorescence as compared to LPS-treated tissue cultures (**Figures 4A–C**). Similar results were obtained, when RT-qPCR analysis for *Tnfa*-mRNA was carried out at the end of each experiment in the same set of tissue cultures (**Figure 4D**). The results demonstrate the anti-inflammatory effects of IL10 in LPS-treated tissue cultures, which is in line with recent reports (28). Thus, we conclude that tissue cultures prepared from our new TNFα-reporter mouse line are suitable tools for studying neural inflammation and anti-inflammatory intervention.

The Effects of LPS and IL10 on TNFα, IL6, IL1β, IL10, and IFNγ Expression in Organotypic Tissue Cultures

An electrochemiluminescent-based protein detection assay was employed to confirm the above results at the protein level. The culturing medium of the vehicle-only controls, LPS, and LPS + IL10 treated tissue cultures prepared from C57BL/6 mice was analyzed for TNFα. Tissue cultures were subjected to further qPCR analysis. **Figure 5A** shows that while TNFα was barely evident in the medium of vehicle-only treated controls, the LPS-treated group demonstrated a significant increase in TNFα.

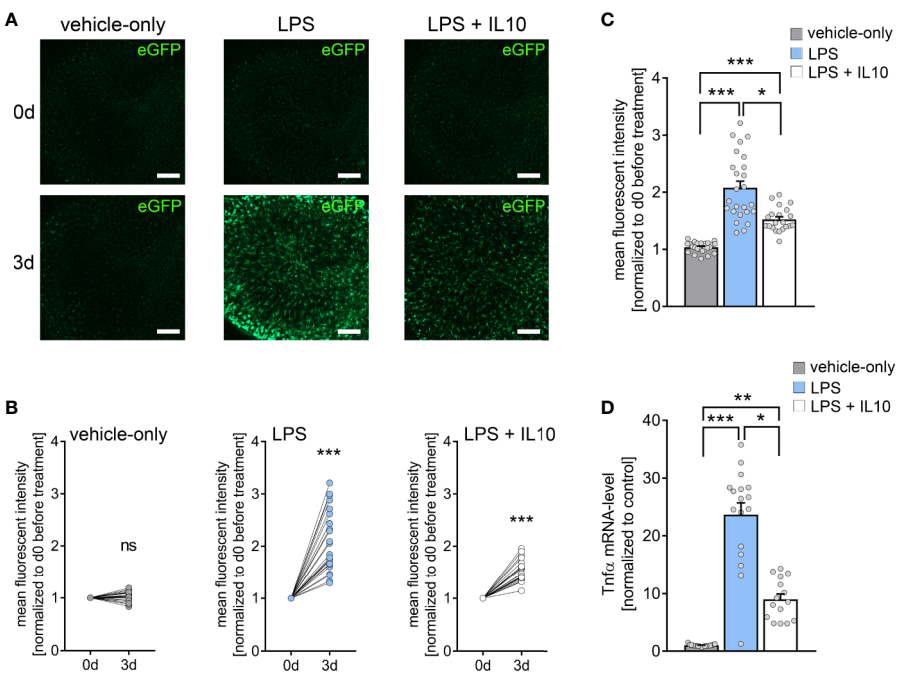

FIGURE 4 | Interleukin 10 (IL10) attenuates *in vitro* LPS-induced TNFα expression. **(A)** Examples of time-lapse imaging of *C57BL/6-Tg(TNFa-eGFP)* tissue cultures before treatment (0 d, row at the top) and 3 days after treatment with vehicle-only, LPS (1 µg/ml) or LPS (1µg/ml) + IL10 (10 ng/ml), respectively (3 d, row at the bottom). Scale bars: 200 µm. **(B, C)** Group data of eGFP fluorescence intensities before the treatment (0 d) and after 3 days in the respective groups ($n_{vehicle\text{-}only}$ = 24, n_{LPS} = 25, $n_{LPS+IL10}$ = 21 cultures per group; Wilcoxon matched-pairs signed rank test, B). IL10 restrains LPS-induced microglial TNFα expression (Kruskal-Wallis test followed by Dunn's multiple comparisons, C). **(D)** Changes in eGFP fluorescence intensity correspond to *Tnfa*-mRNA levels in the respective groups ($n_{vehicle\text{-}only}$ = 17, n_{LPS} = 17, $n_{LPS+IL10}$ = 15 cultures per group; Kruskal-Wallis test followed by Dunn's multiple comparisons test). Individual data points are indicated by colored dots. Values represent mean ± s.e.m. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; ns, not significant difference).

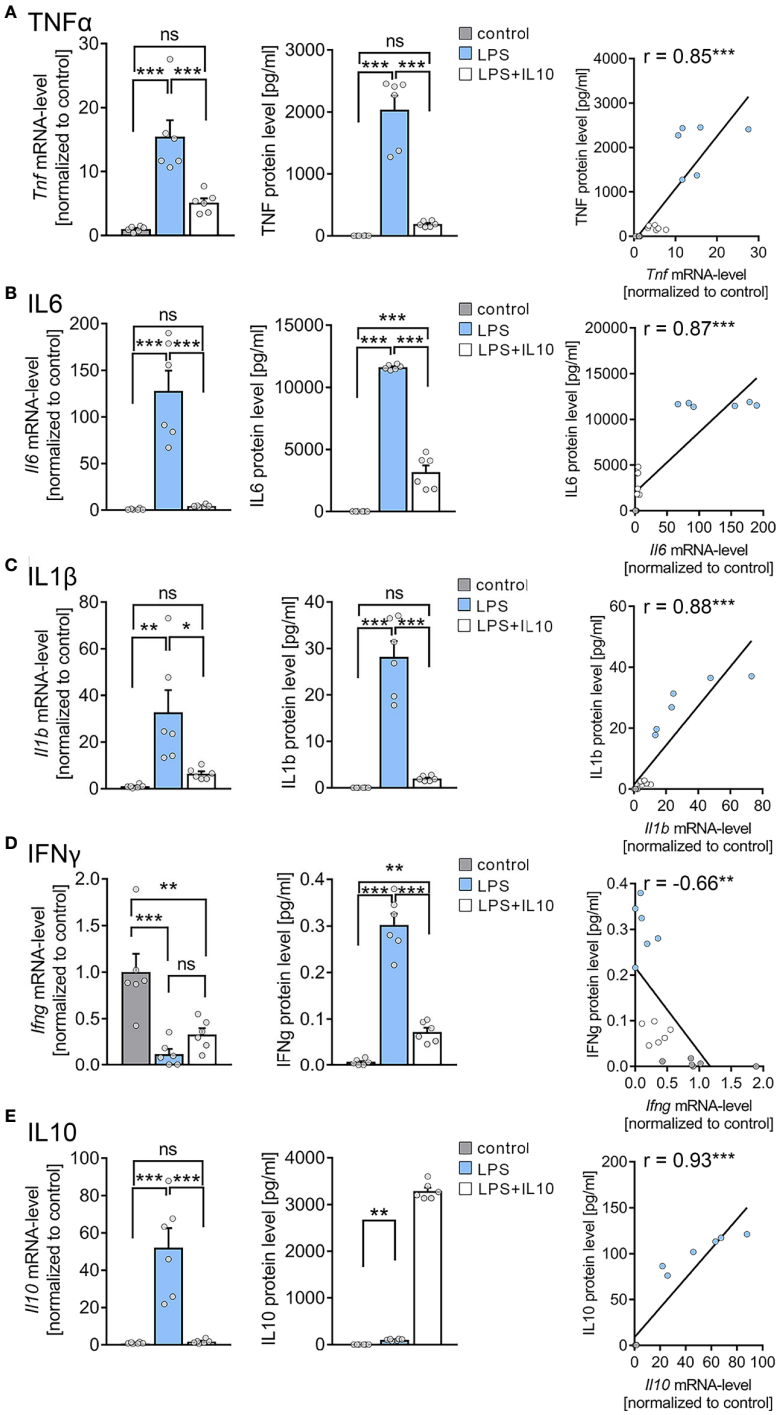

FIGURE 5 | Expression profiles of cytokines in entorhino-hippocampal tissue cultures exposed to Lipopolysaccharide and interleukin 10 (IL10). **(A–D)** Group data and correlations of mRNA and protein levels in the incubation medium of **(A)** tumor necrosis factor α (TNFα), **(B)** interleukin 6 (IL6), **(C)** interleukin 1β (IL1β), and **(D)** interferon γ (IFNγ) in vehicle-only, LPS (1 µg/ml, 3 days) or LPS (1 µg/ml, 3 days) + IL10 (10 ng/ml, 3 days) treated cultures, respectively (n = 6 culture samples or culturing medium respectively for each experimental condition; one-way ANOVA followed by Holm-Sidak's multiple comparison test; linear regression fit indicated by black line; Pearson's r indicated in the figure panels). **(E)** Group data of mRNA and protein levels of IL10 in the respective groups (n = 6 culture samples or culturing medium respectively for each experimental condition; one-way ANOVA followed by Holm-Sidak's multiple comparison test, Mann-Whitney test for statistical comparison of IL10 levels (U = 0); linear regression fit indicated by black line; values for IL10 protein level in the LPS + IL10-treated group were excluded from the fitting procedure; Pearson's r indicated in the figure panels). Individual data points are indicated by colored dots. Values represent mean ± s.e.m. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; ns, not significant difference). U values are provided for significant results only.

Consistent with our mRNA results (all Cq-values are provided in **Supplemental Table S2**), IL10 restrained a significant increase in TNFα protein levels upon exposure to LPS (**Figure 5A**, c.f. **Figure 4D**; for values see **Table S3**). We also tested for the effects of LPS and LPS + IL10 on the pro-inflammatory cytokines IL6, IL1β, and IFNγ and found similar results (**Figures 5B–D**). Specifically, IL6 protein levels increased significantly in the LPS-treated group and this increase was attenuated in the presence of IL10. The anti-inflammatory effect of IL10 was evident in all pro-inflammatory cytokines tested, i.e., IL6, IL1β, and IFNγ. Interestingly, the change in *Ifng*-mRNA levels did not reflect altered IFNγ protein concentrations in the medium. While LPS triggered a reduction in *Ifng*-mRNA, a slight but highly significant increase in IFNγ was detected (**Figure 5D**). From these results, we conclude that organotypic tissue cultures are suitable tools to study LPS-induced neural inflammation and anti-inflammatory treatment strategies, e.g., IL10 administration.

IL10 Levels in Organotypic Tissue Cultures

Next, we examined changes in endogenous *Il10*-mRNA and IL10 protein levels (**Figure 5E**). A significant increase in *Il10*-mRNA levels was detected in the LPS-treated group, which was prevented by the application of recombinant IL10. Consistent with these findings, IL10 protein levels significantly increased in the medium of LPS-treated tissue cultures, and the exogenously applied recombinant IL10 was readily detected in the LPS + IL10-treated group (**Figure 5E**). We conclude, therefore, that an endogenous source of IL10 exists in our tissue cultures. Consistent with a negative-feedback mechanism, this endogenous IL10 source is obstructed by the administration of recombinant IL10. It appears that the endogenous source of IL10 is insufficient to prevent LPS-induced alterations in synaptic plasticity.

IL10 Restores 10 Hz rMS-Induced Synaptic Plasticity in LPS-Treated Tissue Cultures

To address the question of whether IL10 is able to restore rMS-induced synaptic plasticity in LPS-treated tissue cultures, we repeated the experiments described above and stimulated once more tissue cultures with 10 Hz rMS. Basic intrinsic properties (**Figures 6A–C**) and spontaneous excitatory postsynaptic currents (sEPSCs, **Figure 7**) were recorded from CA1 pyramidal neurons.

Figure 6 shows that there was no major difference in input-output properties and no significant difference in the resting membrane potential (RMP) or input resistance between stimulated and non-stimulated tissue cultures (**Figures 6C, D**). Similar results were obtained from tissue cultures treated with LPS (1 μg/ml, 3 days) and LPS (1 μg/ml, 3 days) + IL10 (10 ng/ml, 3 days). Similarly, IL10-only treatment (10 ng/ml, 3 days) had no effects on RMP and input resistances, and an increase in RMP was detected in response to 10 Hz rMS (**Figure S2**). We conclude from these results that 10 Hz rMS has no major effects on basic functional properties of CA1 pyramidal neurons.

Recordings of AMPA-receptor mediated sEPSCs in the same set of neurons showed no significant differences in baseline excitatory synaptic transmission in non-stimulated control cultures (**Figure 7**, Kruskal-Wallis test followed by Dunn's multiple comparisons test; for IL10-only treated cultures see **Figure S2**). Notably, we confirmed the previously reported rMS-induced potentiation of excitatory neurotransmission, and the negative effects of LPS on rMS-induced synaptic plasticity were again observed in these experiments (**Figures 7A, B**). Finally, in the LPS + IL10-treated group, a significant increase in sEPSC amplitudes was detected following rMS (**Figure 7B**). Overall, these results are evidence that the anti-inflammatory cytokine IL10 restores the ability of neurons to express synaptic plasticity probed by 10 Hz rMS in an *in vitro* model of LPS-induced neural inflammation.

DISCUSSION

A substantial body of research supports the fact that inflammatory cytokines and other immune mediators are capable of affecting synaptic transmission and plasticity (25, 27, 30, 44–46). Indeed, bacteremia and sepsis trigger complex responses in the central nervous system including microglia activation and alterations in synaptic plasticity (47, 48). However, the underlying mechanism between microglia activation and synaptic plasticity under physiological and pathological conditions remains poorly understood [c.f., (49)]. The results of the present study support organotypic tissue cultures as tools to study synaptic plasticity under the conditions of neural inflammation and to test for anti-inflammatory and/or plasticity-promoting interventions. Moreover, findings validate the use of tissue cultures prepared from a new transgenic mouse line, i.e., TNFα-reporter mice, and identify microglia as a major source of LPS-induced TNFα and, hence, neural inflammation in our experimental setting. Using this approach, we demonstrate that (i) IL10 restrains LPS-induced neural inflammation *in vitro* and (ii) restores synaptic plasticity probed by rMS in tissue cultures exposed to LPS. These results provide empirical support for the use of rTMS as a diagnostic tool in the context of brain inflammation.

Our previous study demonstrated that the *in vivo* intraperitoneal injection of LPS is accompanied by an increase in brain TNFα levels and alterations in the ability of hippocampal CA1 pyramidal neurons to express excitatory synaptic plasticity (23). Interestingly, in the absence of an LPS-triggered serum cytokine pulse, i.e., when LPS is administered directly to brain tissue (*in vitro)*, similar effects on synaptic plasticity are observed. While baseline synaptic transmission is not affected, the ability to potentiate excitatory neurotransmission is altered in the presence of LPS, both following electric tetanic stimulation and 10 Hz rMS *in vitro*. These findings indicate that LPS can act directly on neural tissue and that it is sufficient to trigger alterations in synaptic plasticity in the CNS. Notably, these effects are not readily explained by LPS-mediated neurotoxicity as shown by the PI stainings in our tissue cultures, and the findings do not support rTMS as affecting cell viability. Yet, the precise role of TLR4-mediated microglia

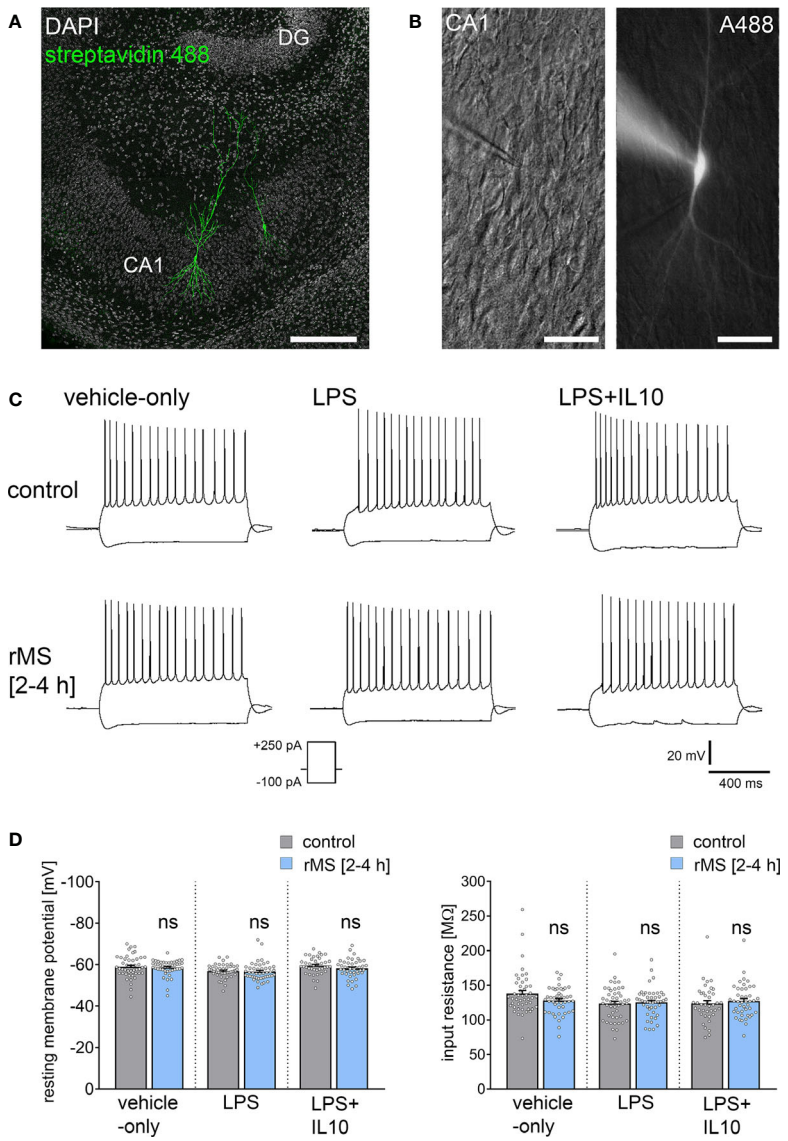

FIGURE 6 | Repetitive magnetic stimulation (rMS) and Lipopolysaccharide have no major impact on intrinsic cellular properties of CA1 pyramidal neurons. **(A, B)** Examples of CA1 pyramidal neurons recorded and filled with Alexa 488 (10 µM). Cytoarchitecture visualized with DAPI in **(A)**, Dot gradient contrast in **(B)**. Scale bar: **(A)** 200 µm, **(B)** 50 µm. **(C)** Sample traces of input-output properties in CA1 pyramidal neurons from vehicle-only, LPS (1 µg/ml, 3 days) or LPS (1 µg/ml, 3 days) + IL10 (10 ng/ml, 3 days) treated tissue cultures, respectively. **(D)** Resting membrane potential and input resistance are not significantly different between the groups (vehicle-only: $n_{control}$ = 47 cells in 8 cultures, $n_{rMS\ [2\text{-}4h]}$ = 46 cells in 8 cultures; LPS: $n_{control}$ = 47 cells in 8 cultures, $n_{rMS\ [2\text{-}4h]}$ = 46 cells in 8 cultures; LPS + IL10: $n_{control}$ = 40 cells in 7 cultures, $n_{rMS\ [2\text{-}4h]}$ = 44 cells in 8 cultures; Mann-Whitney test; one recording excluded in the LPS/rMS-group due to high series resistance during recording). Individual data points are indicated by colored dots. Values represent mean ± s.e.m. (ns, not significant difference).

activation (50) warrants further investigation. It remains to be investigated, for example, whether LPS impairs plasticity via microglia activation, which in turn could trigger TNFα expression (and other cytokines), or via additional direct effects on astrocytes and neurons (51, 52).

The effects of LPS on synaptic plasticity concur with the findings of our recent work on the concentration-dependent effects of TNFα in CA1 pyramidal neurons (30). In this earlier study, we showed that a high concentration of TNFα impairs the expression of excitatory synaptic plasticity, which reflects the LPS effects reported in the present study. It is conceivable, therefore, that TNFα contributed to the detrimental effects of LPS on synaptic plasticity in our experimental setting, which is supported by recent studies on microglial TNFα deficiency (28). However, the precise molecular signaling pathways and neuronal targets through which TNFα (and other pro-inflammatory cytokines) modulate synaptic plasticity, e.g. through modifications in AMPA receptor accumulation at synaptic sites, remain poorly understood. Since IL10 restrains the expression of several pro-inflammatory cytokines, it is

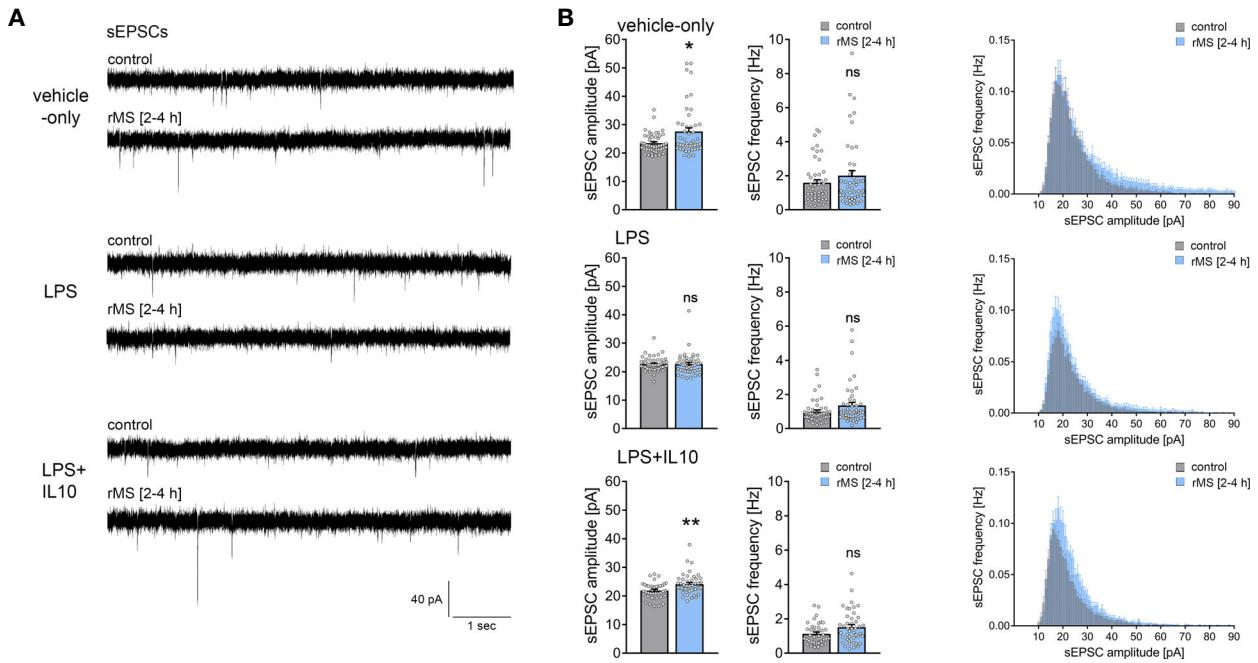

FIGURE 7 | Interleukin 10 (IL10) restores the ability of CA1 pyramidal neurons to express synaptic plasticity in tissue cultures exposed to Lipopolysaccharide. **(A, B)** Sample traces and group data of spontaneous excitatory postsynaptic currents (sEPSCs) recorded from CA1 pyramidal neurons of vehicle-only, LPS (1 µg/ml, 3 days) or LPS (1 µg/ml, 3 days) + IL10 (10 ng/ml, 3 days) treated tissue cultures, respectively. 10 Hz repetitive magnetic stimulation (900 pulses) was used to probe excitatory synaptic plasticity (vehicle-only: $n_{control}$ = 47 cells in 8 cultures, $n_{rMS\ [2\text{-}4h]}$ = 46 cells in 8 cultures; LPS: $n_{control}$ = 47 cells in 8 cultures, $n_{rMS\ [2\text{-}4h]}$ = 47 cells in 8 cultures; LPS + IL10: $n_{control}$ = 40 cells in 7 cultures, $n_{rMS\ [2\text{-}4h]}$ = 44 cells in 8 cultures; Mann-Whitney test, vehicle-only: $U_{sEPSC\ amplitude}$ = 791, LPS + IL10: $U_{sEPSC\ amplitude}$ = 567). Individual data points are indicated by colored dots. Values represent mean ± s.e.m. (* $p < 0.05$, ** $p < 0.01$; ns, not significant difference). U values are provided for significant results only.

conceivable that the plasticity restoring effects of IL10 do not depend exclusively on concentration-dependent effects of TNFα [c.f., (30)]. Considering the results of our protein detection assay, future research should extend this exploration to investigate the role of the concentration-dependent effects of IL6, IL1β, and IFNγ on synaptic plasticity modulation.

Regardless of these considerations, we identified microglia as a major source of LPS-induced TNFα in tissue cultures prepared from our new TNFα-reporter mouse line. Changes in eGFP expression were reflected in *Tnfa*-mRNA expression and altered TNFα protein levels in the culturing medium, thus validating the use of TNFα-reporter mice. Furthermore, the anti-inflammatory effects of IL10 were readily detected using live-cell microscopy and quantification of the eGFP signal [for recent work on the effects of IL10 in the central nervous system see (53–55)]. Hence, tissue cultures prepared from these mice are suitable to monitor neural inflammation and anti-inflammatory interventions *in vitro*. Certainly, crossing *C57BL/6-Tg(TNFa-eGFP)* mice with other suitable transgenic mouse lines [e.g., *HexB-tdTomato*; (56)] could be instrumental to better understand the complex role of microglial TNFα in physiological and pathological conditions (57, 58).

In this context, several recent studies have implicated microglia and defective IL10 signaling in various brain disease and lesion models (59–62). Further, IL10 expression has been reported for cultured microglia (63). Notably, IL10 has recently been linked to the modulation of excitatory neurotransmission (64, 65), suggesting that microglia may affect synaptic plasticity via IL10. However, to date, transcriptome analyses of microglia isolated from various *in vivo* disease models have failed to demonstrate IL10 expression in microglia (28, 66–68). For example, recovery from spinal cord injury depended on IL10, which was produced by monocytes and not microglia (69). Considering that cultured microglia lose their characteristic tissue imprints and adopt a gene expression signature that resembles prototype macrophages (70, 71), it could be assumed that microglia are not capable of producing IL10 in a mature (organotypic) environment. Our experiments in mature, i.e., three-week old tissue culture preparations show that LPS triggers endogenous IL10 expression, both at the mRNA and protein level. However, when recombinant IL10 was administered *Il10*-mRNA expression was reduced to control levels. These findings are consistent with a prominent negative feedback mechanism that regulates IL10 expression in organotypic tissue cultures. It could be considered that the controversy between studies on microglial IL10 expression may depend, at least in part, on such negative feedback mechanisms and, hence, on the presence of other IL10 sources in the respective experimental settings, e.g., the presence of monocytes, T-cells and other peripheral immune cells, which are absent in three-week old tissue cultures. Future studies should explore the relevance of such negative feedback

mechanism and genomic regulatory elements that suppress microglial IL10 production in the presence of IL10 and other restricting factors. We are confident that our organotypic tissue culture approach will be helpful in addressing these and other important questions. Accordingly, testing for the effects of neural activity and brain stimulation in this context will be an interesting avenue for future research. The results of the present study provide robust evidence that non-invasive brain stimulation techniques, i.e., rTMS, are suitable tools for monitoring alterations in synaptic plasticity in brain inflammation and the plasticity restoring effects of IL10.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available from the corresponding author upon reasonable request.

ETHICS STATEMENT

The animal study was reviewed and approved by the animal welfare committee (V54-19c20/15-F143/37), the local animal welfare officers (Universities of Frankfurt, Mainz and Freiburg), and/or the institutional animal care and use committee (Sheba Medical Center).

AUTHOR CONTRIBUTIONS

Roles of authors and contributors have been defined according to ICMJE guidelines and author contributions have been reported according to CRediT taxonomy. Conceptualization: ML, SJ, NM, and AV. Methodology: ML, NM, and AV. Formal Analysis: ML, AE, PK, AS, SR-R, and NM. Investigation: ML, AE, PK, AS, SR-R, and NM. Resources: IG, NY, SF, AW, and AV. Writing Original Draft: ML and AV. Visualization: ML, AE, and AV. Supervision: NM and AV. Project Administration: ML, TD, NM, and AV. Funding Acquisition: ML, TD, SJ, NM, and AV. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.614509/full#supplementary-material

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Developmental Dysfunction of the Central Nervous System Lymphatics Modulates the Adaptive Neuro-Immune Response in the Perilesional Cortex in a Mouse Model of Traumatic Brain Injury

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Rationale: The recently discovered meningeal lymphatic vessels (mLVs) have been proposed to be the missing link between the immune and the central nervous system. The role of mLVs in modulating the neuro-immune response following a traumatic brain injury (TBI), however, has not been analyzed. Parenchymal T lymphocyte infiltration has been previously reported as part of secondary events after TBI, suggestive of an adaptive neuro-immune response. The phenotype of these cells has remained mostly uncharacterized. In this study, we identified subpopulations of T cells infiltrating the perilesional areas 30 days post-injury (an early-chronic time point). Furthermore, we analyzed how the lack of mLVs affects the magnitude and the type of T cell response in the brain after TBI.

Methods: TBI was induced in K14-VEGFR3-Ig transgenic (TG) mice or in their littermate controls (WT; wild type), applying a controlled cortical impact (CCI). One month after TBI, T cells were isolated from cortical areas ipsilateral or contralateral to the trauma and from the spleen, then characterized by flow cytometry. Lesion size in each animal was evaluated by MRI.

Results: In both WT and TG-CCI mice, we found a prominent T cell infiltration in the brain confined to the perilesional cortex and hippocampus. The majority of infiltrating T cells were cytotoxic CD8+ expressing a $CD44^{hi}CD69+$ phenotype, suggesting that these are effector resident memory T cells. K14-VEGFR3-Ig mice showed a significant reduction of infiltrating CD4+ T lymphocytes, suggesting that mLVs could be involved in establishing a proper neuro-immune response. Extension of the lesion (measured as lesion volume from MRI) did not differ between the genotypes. Finally, TBI did not relate to alterations in peripheral circulating T cells, as assessed one month after injury.

Conclusions: Our results are consistent with the hypothesis that mLVs are involved in the neuro-immune response after TBI. We also defined the resident memory CD8+ T cells as one of the main population activated within the brain after a traumatic injury.

Keywords: controlled cortical impact (CCI), meningeal lymphatic vessels, CD8+ T lymphocytes, resident memory T cells, Chronic Traumatic Brain injury

INTRODUCTION

Traumatic brain injury (TBI) is among the top causes of death and disability in adult life (1, 2). It is estimated that at least 70 million people worldwide incur TBI every year (3). The number of prevalent cases (as reported for 2016) is above 55 million, with patients suffering from a wide range of lifelong physical and psychological invalidities (4).

TBI is defined as an alteration in brain function, or other evidence of brain pathology, caused by an external force (5), which results in immediate neuronal cell death, diffuse axonal injury, ischemia, and hemorrhage (6). These primary insults initiate a progressive cascade of secondary injuries, which include macrophage infiltration (7), neuro-inflammation (microglia and astrocyte activation associated with cytokine production), edema formation, oxidative stress, neuronal necrosis and apoptosis, and white matter atrophy (6). Secondary injuries can progress for years in patients and rodent models of TBI, and cause neurological and psychiatric deficits associated with the pathology (8).

Recruitment of peripheral immune cells, including T lymphocytes, into the brain is among the secondary events that have been described following TBI (9–12). Two distinct waves of infiltrating CD3+ T cells have been reported in the injured brain. First, a massive infiltration immediately commences after trauma and peaks 3 days post-injury (dpi) (9). After one month, there is a late adaptive immune response with a second recruitment, which persists chronically (11). However, the mechanisms and the consequences of the activation of the adaptive immune system after TBI are still poorly understood.

A proper immune surveillance of the brain was long disputed (13), due to the lack of a classical lymphatic system within the central nervous system (CNS). However, recent studies have described the presence of anatomically distinct lymphatic vessels in the meninges surrounding the brain and the spinal cord. These meningeal lymphatic vessels (mLVs) preferentially drain the cerebrospinal fluid together with cells and macromolecules into the deep cervical lymph nodes (dcLNs) (14–17). Within these secondary lymphoid organs, brain-derived antigens are presented to resident T lymphocytes, evoking different cellular fate and immune responses based on the inflammatory milieu. It has been demonstrated that dcLNs, together with superficial cervical LNs (scLNs), play a specific role in neuro-immune interaction, ensuring the protection of brain cells by promoting a non-cytotoxic immune response (18–20). From this prospective, mLVs and dcLNs are essential components of a putative specific CNS lymphatic system, and the mLVs could be essential in the activation of immune responses to brain insults.

The aim of our work is to better characterize the late adaptive immune response and to decipher the mechanisms underpinning the activation of T lymphocytes after TBI, focusing on the specific role of mLVs in this process. In this regard, we induced a cerebral contusion in the cortex of transgenic K14-VEGFR3-Ig (TG) mice that completely lack lymphatic vessels in several tissues, including the meninges (16, 21, 22). One month after brain injury, infiltrating T lymphocytes and circulating peripheral T cell populations in the spleen were phenotyped by flow cytometry. MRI was used to evaluate lesion size by comparing TG animals to their wild type (WT) littermates. We determined the persistence of putative resident memory cells mediating a CD8+ cytotoxic immune response in the perilesional cortical areas after TBI. We further demonstrate that a functional mLVs are important for the neuro-immune interaction after TBI, and the lack of mLVs results in the imbalance of the evoked T cell immune response. Our data also show that the TBI-elicited response in the CNS is specific, and that the analysis of the systemic immunity does not reflect the immune activation observed within the brain. No differences in MRI cortical lesion were found between the two genotypes. We suggest that the brain resident memory T cells, presenting an effector phenotype, are part of the cellular components characterizing the secondary injuries after TBI.

MATERIAL AND METHODS

Mice

Initial breeding pairs of K14-VEGFR3-Ig mice [C57BL/6JOlaHsd background (21)] were transferred from the University of Helsinki, and the colony was further expanded and maintained at University of Eastern Finland (Kuopio, Finland). Wild type and transgenic K14-VEGFR3-Ig mice used in all the experiments were littermates. Genotype screening was routinely confirmed by polymerase chain reaction analysis of ear punch samples. Mixed WT and TG mice were housed in standard laboratory cages (four animals per cage, until surgery) in a controlled enriched environment (constant temperature, 22 ± 1°C, humidity 50–60%, lights on 07:00–19:00), with food and water available *ad libitum* (23). After TBI induction, mice were kept two per cage, separated individually by a pierced partition. All animal procedures were approved by the Animal Ethics Committee of the Provincial Government of Southern Finland (ESAVI-2018-008787) and performed in accordance with the guidelines of the European Community Council Directives 2010/63/EU.

Controlled Cortical Injury Mouse Model of Traumatic Brain Injury

All surgical procedures were performed aseptically whenever possible. Adult, 5 month-old male mice were deeply anesthetized with isoflurane (5% for induction, 1.0–1.5% for maintenance, in 0.5 L/min air; see **Supplementary Table 1**), injected with Carprofen (4 mg/Kg; s.c.) and the heads fixed to a stereotaxic frame (Kopf, Tujunga, USA). The scalp was shaved and then scrubbed (3x) with Betadine (povidone-iodine 10%) and 70% ethanol alternately, then local anesthesia of 2% Xylocain gel was applied. After skull exposure, a 5 mm circular craniotomy was manually drilled over the left parieto-temporal cortex, with the posterior edge of the craniotomy apposed to the lambdoid suture and the right edge to the sagittal suture. In order to reduce heating during manual craniotomy, the skull was irrigated with cold 0.9% saline solution. The carved bone was carefully removed, without disrupting the underlying dura, and placed in 1% Betadine solution. Thereafter, the animal was disconnected from isoflurane anesthesia for 5 min [stage 3 plane 1 according to Guedel's classification (24)], and CCI was induced using an electromagnetic stereotaxic impact actuator (ImpactOne, Leica, Richmond, VA, USA). The 3 mm blunt tip of the impactor was adjusted to the center of the exposed dura perpendicular to the brain surface, and the impact was administered at a depth of 0.5 mm, speed of 5.0 m/s, and dwell time of 100 ms. The total duration of the craniotomy procedure including anesthesia induction was 35–40 min (**Supplementary Table 1**). After the impact, the mouse was reconnected to the isoflurane system and the skull secured with bone cement (Selectaplus + Palacos R+G 50/50). The scalp was sutured and treated with Cicatrene powder (Neomycin + Bacitracin) and Terramycin spray (Oxytetracycline). The total duration of post-impact surgery was 10 min. The mice were injected i.p. with 1 ml pre-warmed sterile saline (35°C) and allowed to fully recover in an incubator at 32°C. Mice were followed for the subsequent 48 h for any signs of illness or distress, in which case Carprofen was administered. Daily examination was performed for general health/mortality and morbidity for the rest of the study. No mortality was observed.

Craniotomy-related neuroinflammation has been previously reported in this model and the craniotomy itself (surgery) can be considered a form of mild brain trauma (25, 26). Moreover, CCI is a model of penetrating injury, involving dura damage, which has a severity that bypasses the possible effect of meningeal inflammation related to the craniotomy. The aim of our study is to characterize the adaptive immunity in response to a moderate TBI. Therefore, we did not analyze how differences in trauma severity (*i.e.*, CCI *vs.* sham-surgery) can affect the neuro-immune response. In compliance to the 3R principle, we excluded the sham-operated animals and used naïve mice not exposed to the surgical procedure as proper controls.

In Vivo Magnetic Resonance Imaging and Lesion Volume Definition

MRI data were acquired 21 days after TBI induction in a 7T horizontal magnet (Bruker Pharmascan, Ettlingen, Germany). Images were acquired using a four-channel mouse brain surface coil, a 3D T2-weighted Fast Spin-Echo sequence (RARE, repetition time 1.5 s, effective echo time 48 ms, 16 echoes per excitation) with 100 μm isotropic resolution (field of view 25.6 mm x 128.8 mm x 9.6 mm; acquisition matrix 128 x 256 x 96). Scans were performed with the mouse under 1.0–1.5% maintenance isoflurane anesthesia (70/30 N_2O/oxygen gas mixture, 1 L/min). The average acquisition time was 40 min, including anesthesia induction. A pressure sensor was used to monitor the respiratory rate, and respiratory gating was used to minimize motion artifacts.

T2-weighted images were used to evaluate the extent of the lesion (**Figure 5**, and **Supplementary Figures 4** and **5**). Regions of interest (ROIs) were outlined for volumetric analysis, avoiding the brain-skull interface and ventricles, throughout the entire extension of the brain (excluding olfactory bulbs and cerebellum). Lesion was defined as cortical/subcortical areas with hyper-intense signal (cystic lesion) and/or signal void areas (tissue cavity) from T2-weighted images (27, 28). Volumes of the lesion and of the ipsilateral and contralateral hemispheres were measured using Aedes (http://aedes.uef.fi), an in-house written MatLab program (MathWorks, Natick, MA). The lesion volume and the volumes of ipsilateral and contralateral healthy hemispheres were calculated from 80 consecutive slices in the coronal plane and adjusted in the sagittal plane (66 slices) and in the axial plane (99 slices) with a volume resolution of 200 x 500 x 100 μm.

Quantification of Brain Contusion Area and Brain Atrophy

Measured volumes from MRI analysis were used to quantify the volume of the brain contusion and the brain atrophy, as previously described (29, 30). The relative percentage of infarct volume was calculated using the following formula:

$$contusion\ volume\ (\%) = \frac{Vc - (Vi - Vl)}{Vc} * 100$$

and brain atrophy was determined as:

$$brain\ atrophy\ (\%) = \frac{(Vi - Vc)}{Vc} * 100$$

where Vc = volume of contralateral hemisphere; Vi = volume of ipsilateral hemisphere; and Vl = measured lesion volume.

Analysis was performed blinded to the study groups. The contusion volume was measured from 22 TBI mice from the following experimental groups: WT–CCI, n = 13; and TG–CCI, n = 9. Analyses of contusion volume and brain atrophy progression at day 3 and day 14 has been conducted on *ad hoc* prepared mice: WT–CCI, n = 4; and TG–CCI, n = 4.

Cell Isolation of Leukocytes

Thirty days after TBI induction, mice were anesthetized with an overdose of Avertin (Sigma, St. Louis, MO, USA) then transcardially perfused with ice-cold heparinized saline (6 min, 6 ml/min). Brains were collected and placed on ice in calcium

and magnesium-free Hanks Balanced salt solution (HBSS) with 25 mM HEPES (both from Sigma).

Based on the analysis of MRI, we defined *a priori* the mean extension of the lesion and of the perilesional areas for all the TBI mice. Brains were sliced using a 1 mm scored matrix (Zivic Instruments, Pittsburgh, PA, USA): 6 mm thick coronal cut encompassing the lesion area was split along the central sagittal axis into left injured and right uninjured sides. Cortical areas enclosed between the rhinal and the sagittal sulci, and the corresponding hippocampi, were further isolated, pooled together, and placed in HBSS+HEPES. From the injured sides, penetrated cortical areas were visually identified (lesion area - **Supplementary Figure 1**) and carefully excised along the lesion ridge to pick only the perilesional cortex for further purification of leukocytes.

Brain samples were minced with scissors and then incubated at 37°C on a roller for 30 min in digest buffer containing 1.25 mg/ml Collagenase Type 4 (Worthington, Lakewood, NJ, USA) and 100 U/ml DNAseI (Sigma) in DMEM with GlutaMAX (Gibco Thermo Fisher Scientific, Waltham, MA, USA). Samples were filtered through a 100 μm cell strainer (Corning, Weisbaden, Germany), and centrifuged at 600 x g for 5 min. Myelin debris was removed using Debris Removal Solution (Miltenyi Biotech, Bergisch Gladbach, Germany) according to the manufacturer's protocol. Briefly, cells were resuspended in ice-cold Dulbecco's phosphate buffered saline (D-PBS, Sigma) with Debris Removal Solution, then overlaid with ice-cold D-PBS and centrifuged at 2,500 x g for 10 min at 4°C. Supernatant including myelin layer was carefully removed leaving the clear phase and the pellet. Samples were washed in ice-cold D-PBS, centrifuged at 600 x g for 10 min at 4°C, and the recovered pellets were stained directly for flow cytometry.

Spleens and dcLNs were separately collected in ice-cold HBSS+HEPES and each processed by crushing through a 70 μm cell strainer (Corning). dcLNS were washed with ice-cold D-PBS containing 1% bovine serum albumin (BSA) and 2 mM ethylenediaminetetraacetic acid (EDTA), centrifuged 500 x g for 10 min and resuspended in RPMI-1640 (all from Sigma). Crushed spleens were washed with ice-cold HBSS+HEPES, centrifuged 500 x g for 5 min before red blood cells were lysed in 1X PharmLyse (BD Biosciences, San Jose, CA USA) for 8 min at room temperature (RT). Lysed cells were washed with HBSS+HEPES, centrifuged as above, resuspended in RPMI-1640 (Sigma), and counted on a Bürker grid hemocytometer.

Flow Cytometry Staining and Analysis

Spleen cells (500,000 per mouse), and total cells isolated from dcLNs and brain were each stained separately. Cells were first washed with D-PBS, and centrifuged at 400 x g for 5 min. The supernatant was removed, and then Zombie NIR fixable viability dye (1:1,000 BioLegend, San Diego, CA, USA) was added for 15 min at RT. Without washing, CD16/32 FcR block (5 μg/ml, BD Biosciences) was added followed by the appropriate antibody mix. Antibodies used: TCRβ PE-Cy7 (1:100 or 1:200 clone H57-597), CD44 PE (1:300 clone IM7) (both BioLegend); CD8a APC-R700 (1:150 or 1:200, clone 53-6.7), CD69 BV421 (1:100, clone H1.2F3), CD25 BB515 (1:150, clone PC61) (BD Biosciences); CD4 FITC (1:500, clone RM4-5), CD4 eFluor506 (1:500, clone RM4-5), CD8 PerCP eFluor710 (1:300, clone 53-6.7), CD44 APC (1:300 or 1:400, clone IM7), FoxP3 (1:40, clone FJK-16s) (eBioscience Thermo Fisher Scientific, Waltham, MA, USA); CD69 APC (1:20, clone H1.2F3, Miltenyi Biotech). All antibodies were used at titers determined empirically under experimental conditions.

Cells were incubated for 30 min at 4°C. Afterwards, samples were washed twice in HBSS with 1% FBS and then run on FACSAriaIII (BD Biosciences) equipped with 488 and 633 nm lasers, or on CytoFLEX S (Beckmann Coulter) equipped with 405, 488, 561, and 638 nm lasers, both with standard configuration. Compensations were made using OneComp and UltraComp Beads for antibody fluorescence (eBioscience Thermo Fisher Scientific) and ArC amine reactive beads for viability dye (Molecular Probes, Eugene, Oregon, USA). Fluorescent-Minus-One (FMO) controls were made to ensure gating. These control samples contained all antibodies except one to display fluorescent spreading error of compensated data in each channel (31). Data were analyzed using FCSExpress v5 (Denovo Software, Los Angeles, CA, USA) and FlowJo v10.4 (Treestar, Portland, OR, USA). The gating strategy used for the flow cytometry analysis of brain-isolated immune cells is reported in **Supplementary Figure 1**.

CD3 Immunohistochemical Staining

Three mice per genotype were injured and sacrificed 30 days after TBI for the immunohistochemical (IHC) estimation of T lymphocyte localization in the brain. Mice were transcardially perfused with ice-cold NaCl 0.9% followed by 4% PFA. Brains were dissected and post-fixed in 4% PFA by immersion for 24 h at 4°C. Thereafter, specimens were cryoprotected by incubation in 20% glycerol [in 0.02 M potassium phosphate-buffered saline (KPBS), pH 7.4] for 48 h, frozen in N-pentane (3 min at -60°C), and stored at -70°C until sectioning. Frozen coronal sections were cut 25 μm with a sliding microtome, and collected in solution containing 30% ethylene glycol, 25% glycerol in 0.05 M phosphate buffer (PB) and stored at -20°C until further processing. Three sections per brain (approx. 700 μm apart, encompassing the antero-posterior extension of the lesion), were used to estimate the localization of CD3+ infiltrating T lymphocytes by IHC. Floating sections were washed in three changes of 1X PBS before being incubated for 1 h at RT in blocking solution [2% normal goat serum, 1% bovine serum albumin (BSA) 0.1% Triton X-100 and 0.05% Tween20 in PBS]. Sections were incubated overnight at 4°C with rat anti-mouse CD3ε (1:500, clone 17A2, eBioscience Thermo Fisher Scientific) and mouse anti-GFAP (1:500, Sigma) in staining buffer PBS with 1% BSA and 0.05% Triton X-100. After washing 3x with PBS, sections were incubated with conjugated goat secondary antibody anti-rat Alexa Fluor 647 and anti-mouse Alexa Fluor 546- in above staining buffer for 1 h at RT (Respectively 1:500 and 1:250, both Thermo Fisher Scientific). Finally, the sections were washed 3x in PBS and 10 min in 1X PB and mounted on Superfrost Plus slides (Thermo Scientific) with Vectashield containing DAPI (Vector Laboratories, Burlingame, CA, USA). Panoramic photomicrographs of the stained sections were

captured using 20X objective with a fluorescence microscope (Zeiss Observer.Z1), and high-resolution Z-stack images were captured using 20X objective with a confocal microscope (Zeiss LSM710). ZEN 2012 software (Carl Zeiss GmbH) was used for image processing.

Microtubule-Associated Protein 2, NeuN, and Glial Fibrillary Acidic Protein Staining and Analysis

Three sections located at bregma level +0,02, -2,06, and -4,04 mm (corresponding to the anterior and posterior edges and to the center of the lesion site) were selected from the previously sliced brains and stained for the Microtubule-Associated Protein 2 (MAP2; neuronal dendrites), the neuronal antigen NeuN, and the Glial Fibrillary Acidic Protein (GFAP; Type III intermediate filaments in astrocyte). For immunofluorescence procedure, sections were washed in blocking solution (4% BSA, 0,2% Triton X-100 in PBS) for 1 h at RT, followed by overnight incubation at 4°C with the following primary antibodies diluted in blocking solution: mouse anti-GFAP (1:500, Sigma G3893), guinea pig anti-NeuN (1:500, Millipore ABN90), rabbit anti-MAP2 (1:300, Abcam ab32454). After washing in PBS, sections were incubated for 2 h at RT with secondary fluorescent antibodies in blocking solution: Alexa Fluor 546-conjugated goat anti mouse (1:250), Alexa Fluor 488-conjugated goat anti rabbit (1:250), Alexa Fluor 633-conjugated goat anti guinea pig (1:200 all from Invitrogen, Thermo Fisher Scientific). Next, sections were washed in PBS before being mounted onto glass slides and coverslipped using Fluoromount-G (Thermo Fisher Scientific).

Image acquisition was performed using Zeiss Axio Observer Z1 microscope, equipped with a Zeiss AxioCam MR R3 camera, mounting a 10x lens to obtain images from whole-brain sections. Magnification images of infiltrating T cells (**Figure 1**) were acquired using a Zeiss LSM710 confocal microscope, mounting a 25x LCI plan objective (340 x 340 µm, 21 Z-stack slices/image, 20 µm total thickness).

Image analysis was performed using ImageJ software. ROIs were manually selected on images taken from each stained section. After background subtraction, the mean gray value was measured within each ROI (32).

Statistical Analysis

Data Exclusion Criteria

We conducted eight independent experiments, where a total of n = 16 "WT CCI"; n = 12 "WT naïve"; n = 13 "TG CCI" and n = 10 "TG naïve" mice have been analyzed.

Before statistical analysis, brain-derived samples were checked for their quality, based on total T cell recovery. Each sample has been considered independently, and we evaluated the T cell viability and the total number of T cells recovered. Brain samples where T cell viability was below 75% or the total number of live T cells was below 100 counts were *a priori* excluded from the analyses.

Considering two genotypes (WT and TG) and three experimental conditions (T cells infiltrating the brain tissue ipsilateral to the lesion – "ipsi"; T cells infiltrating the tissue contralateral to the lesion – "contra", and T cells from naïve brain tissue – "naïve"), a total of n = 12 "WT ipsi"; n = 7 "WT contra"; n = 5 "WT naïve"; and n = 10 "TG ipsi"; n = 7 "TG contra"; n = 9 "TG naïve" were finally used for statistical analyses.

T cell viability >90% was used for the quality requirement of spleen and dcLN samples. Moreover, we excluded spleen samples presenting more than 50% of necrotic tissue (defined as dark red non-perfused area in the spleen). Considering two genotypes (WT and TG) and two experimental conditions (CCI and naïve), a total of n = 13 "WT CCI"; n = 12 "WT naïve"; and n = 11 "TG CCI", n = 9 "TG naïve" spleens were used for subsequent statistical analyses. Deep cervical lymph nodes have been analyzed in n = 4 "WT CCI" and n = 6 "TG CCI" mice.

Statistical Analysis of Brain- and dcLNs-Related Data

Due to the small amount of T lymphocytes in naïve brains, brain samples were fully acquired on the flow cytometer, and for each population we analyzed both the absolute counts and the percentage referred to the respective parent population. Statistic models were applied considering the nature of our data (counts or percentages) and the experimental groups analyzed. A binomial negative regression was applied to assess statistical differences in the counts of total T cells, of CD4+, and of CD8+ cells between the two genotypes or within the same genotype between independent data. The binomial negative regression considered both genotype and treatment and their interaction. Because data from ipsi and contralateral brain sides are dependent within the same genotype, a linear mixed model was used to evaluate the differences in the total number of CD4+ and CD8+ T lymphocytes between "WT ipsi" vs. "WT contra" or "TG ipsi" vs. "TG contra". As the data were not normally distributed (Shapiro-Wilk test p-value < 0.05), statistical differences between independent data in CD4+ and CD8+ T cell populations (expressed as percentage of T cells), as well as in the percentages of respective subpopulations expressing CD44 and/or CD69 antigens, were analyzed performing the Kruskal Wallis test. Dependent data within the same genotype (ipsi vs. contra) were analyzed performing the paired samples Wilcoxon signed ranked test. In all tests, Bonferroni correction was used to adjust p-values in multiple comparison.

Statistical Analysis of Data From Spleen

All data from spleen are expressed as percentage of the parent population. After establishing the normal distribution of the data (as well as skewness and kurtosis by D'Agostino K-squared test), statistical differences were analyzed performing the Kruskal Wallis test or the paired samples Wilcoxon signed ranked test, depending on the nature of the data (independent or dependent), followed by Bonferroni adjustment.

Statistical Analysis of Magnetic Resonance Imaging Data

The differences in contusion volume and in brain atrophy were analyzed performing the Kruskal Wallis test (21 dpi) or using a

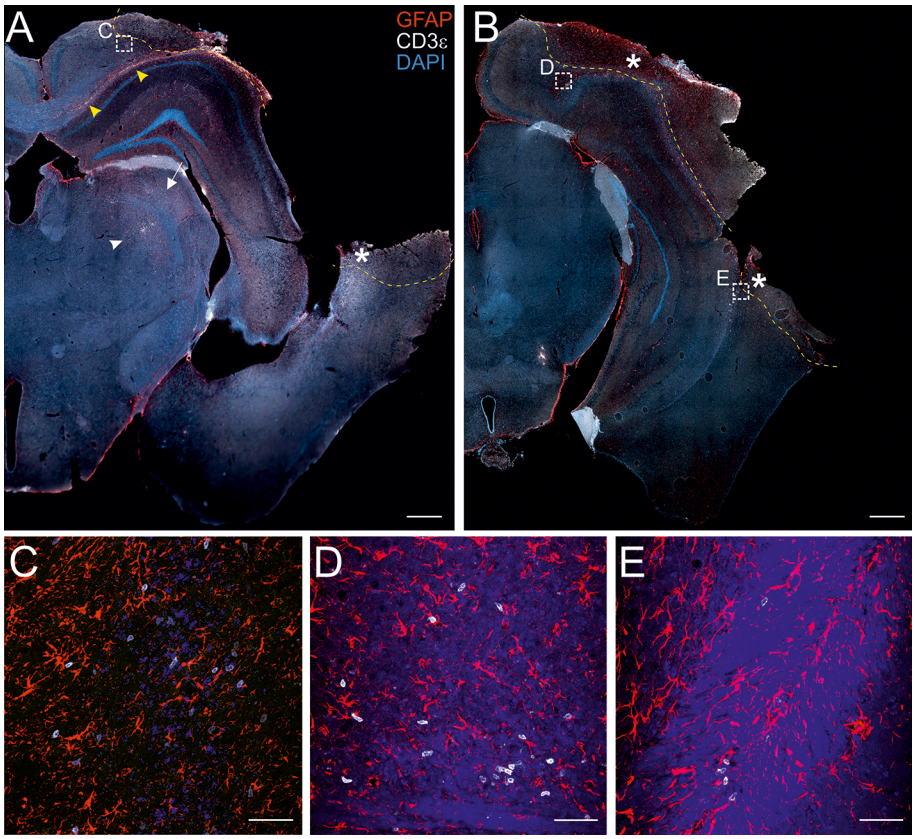


FIGURE 1 | Localization of CD3+ T cells in the perilesional cortices. Representative images of brain sections from CCI mice stained for anti-CD3e (T lymphocytes; white) and anti-GFAP (astrocytes; red), 30 dpi **(A, B)**. The lesion edges in each section are marked with a segmented yellow line. T cells are present within the lesion (star in A and B), in the perilesional cortex (box in A and B, and panel C) and in the corpus callosum (yellow arrow heads in A). CD3+ cells were also observed in the striatum (white arrow in A) and in the thalamus (white arrow head in A). Both scattered cells and presumably-encephalitogenic clusters of T cells (panel **D**) were found within the parenchyma. Panels **(C)** represent a magnification of the area depicted within the white boxes in A. Panels **(D, E)** represent a magnification of the areas depicted within the white box in B. CD3e: white; GFAP: red; DAPI: blue. (A and B, scale bar = 500 μm; C-E, scale bar = 50 μm).

linear mixed model to evaluate the differences between day 3 and day 14 post-TBI. Correlation between TBI-related tissue loss and infarct volume was analyzed by Pearson linear regression, after checking for normal distribution of data as described above.

Statistical analyses were performed using R v3.5.3 software/computing environment (The R foundation for statistical computing). All software packages (MASS, psych, agricolae, multcomp, and lme4) (33–37) were taken from the Comprehensive R Archive Network mirror sites (CRAN; http://CRAN.R-project.org/package=boot). Significance was accepted at the level of $p < 0.05$.

RESULTS

T Cells Preferentially Infiltrate the Cortical Areas Ipsilateral to the Lesion

The presence of infiltrating T lymphocytes in the parenchyma is a signature of brain lesion. At a chronic time point after TBI, we localized the T cell presence in the area of injury and in other brain areas not directly affected by the penetrating injury. For this purpose, we stained brain sections of both WT and TG mice at 30 days post-injury (dpi) for the presence of CD3, a specific marker of T lymphocytes. As expected, T cells are present within the boundaries of the injured area (**Figures 1A, B**). CD3+ cells are also spread throughout the cortical parenchyma, both in proximity to the lesion core (**Figure 1C**) and in more distal areas ipsilateral to the lesion along the cortical layers. Positive immunostaining was also found along the corpus callosum (**Figures 1A, B** and magnification in D), the striatum, the hippocampus, and the thalamus ipsilateral to the lesion (**Figure 1A**). Dim CD3+ signal was present in the contralateral hemisphere, indistinguishable from non-injured mice (data not shown). There was no evident difference in T cell distribution between WT and TG mice: unevenly scattered T cells (**Figures 1C, E**) and T cell clusters (**Figure 1D**) were both observed within the parenchyma, in the perilesional areas.

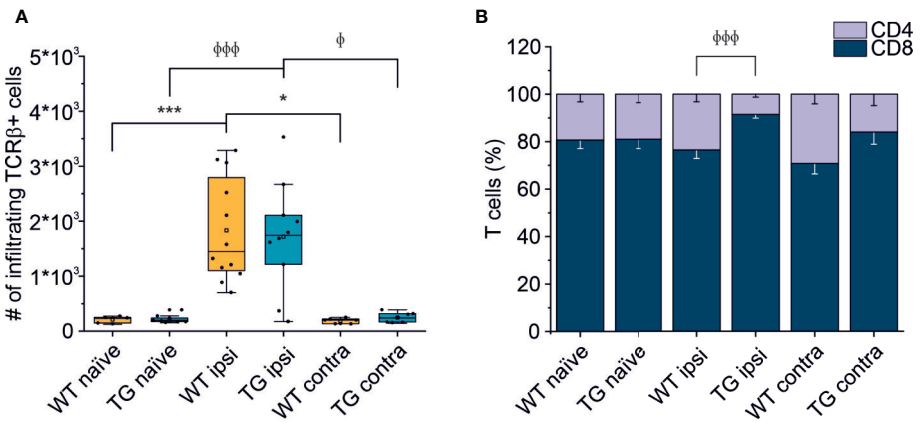
Next, we quantified and characterized the populations of infiltrating T lymphocytes using flow cytometry, focusing on the neo-cortical areas (cortices and hippocampi), excluding the

lesion area, which is characterized by a dysregulated entrance of immune cells (38).

Thirty days after brain trauma induction in TG and littermate WT mice, leukocytes were purified separately from the perilesional and the contralateral cortices (or from the cortex of both WT and TG naïve mice). T cells were identified by staining for T cell receptor (TCRβ) and the presence of the co-receptors CD4 and CD8. Live T cell counts per experimental condition is reported in **Figure 2**. A significant ~10-fold increase of infiltrating T cells was found in both WT (median = 1,449; Q3-Q1 = 1,692) and TG (median = 1,741; Q3-Q1 = 892) mouse brains in the perilesional cortices, compared to corresponding naïve non-injured mice (WT naïve: median = 242; Q3-Q1 = 105; TG naïve: median = 197; Q3-Q1 = 66; for statistical analysis, see **Figure 2C**). In the cortices contralateral to the lesion, the number of TCRβ+ cells did not different from naïve brains (WT contra: median = 201; Q3-Q1 = 84; TG naïve: median = 239; Q3-Q1 = 155; for statistical analysis, see **Figure 2C**). No genotype-related differences were observed (**Figure 2A**).

Perilesional-Infiltrating T Cells Have a Predominant CD8+ Phenotype, and the Constitutive Lack of mLVs Is Associated with a Depressed CD4-Mediated T Cell Response

We next analyzed the CD4:CD8 ratio within the infiltrating T cells (**Figure 2B**) and found a prevalence of CD8+ T cells in all the experimental conditions, regardless of the presence of brain injury. However, limited to the perilesional cortex of TG mice, we detected a significant skew of the CD4:CD8 ratio towards CD8+ cells (CD4:CD8 ratio TG ipsi = 0.097 ± 0.053; WT ipsi = 0.350 ± 0.197; ChiSq: 8.836, mean ranks: 5.50/13.27, p = 8e-04),



C

TCRβ+ cells (counts)					
Comparisons (within WT)	p-value	Comparisons (within TG)	p-value	Comparisons (between genotypes)	p-value
ipsi *vs.* naïve	<0.0001 (***)[a]	ipsi *vs.* naïve	<0.0001 (¤¤¤)[a]	naïve (WT *vs.* TG)	1.0000[a]
ipsi *vs.* contra	0.0112 (*)[b]	ipsi *vs.* contra	0.0151 (¤)[b]	ipsi (WT *vs.* TG)	1.0000[a]
contra *vs.* naïve	1.0000[a]	contra *vs.* naïve	1.0000[a]	contra (WT *vs.* TG)	1.0000[a]

[a] by Kruskal Wallis test, followed by Bonferroni correction
[b] by paired samples Wilcoxon signed rank test, followed by Bonferroni correction

FIGURE 2 | T cell brain infiltration is confined to the perilesional cortices, 30 dpi. Box plot representing the number of infiltrating T cells, defined by expression of TCRβ **(A)** and stacked bargram representing the percentage of CD4+ and CD8+ T cells **(B)** in the brain of WT and TG mice, as analyzed in the perilesional and contralateral cortices (ipsi and contra, respectively; WT ipsi, n = 12; WT contra, n = 7; TG ipsi, n = 10; TG contra, n = 7), or in intact cortices from respective naïve mice (WT naïve, n = 5, TG naïve, n = 9). Independently from the genotype, a significant infiltration of TCRβ+ T cells was observed in the perilesional areas but not in the contralateral hemispheres (comparable to naïve non-injured brains). The majority of brain-infiltrating T cells presented a CD8 phenotype. In the TG CCI mice, there was a significant skew of CD4/CD8 ratio towards CD8+ T cells. Table **(C)** summarizes the results of the statistical analysis in T cell counts between the experimental groups. In **(A)** boxes represent the 25–75% value range, including the median value, indicated with the line. Whiskers represent 1.5x standard deviation (SD). □ indicates the mean value. In the stacked bargram, data are presented as mean ± standard error of the mean (s.e.m.). A binomial negative regression or a linear mixed model was applied to assess statistical differences in the counts of total T cells. The Kruskal Wallis test or the paired samples Wilcoxon signed ranked test was used for the analysis of CD4 and CD8 frequency distribution. $^{\varphi}p < 0.05$ and $^{\varphi\varphi\varphi}p < 0.001$ vs. TG ipsi. *p < 0.05 and ***p < 0.001 vs. WT ipsi. In all tests, Bonferroni correction was used to adjust p-values in multiple comparisons.

while the ratio in the contralateral cortex did not differ between the two genotypes (CD4:CD8 ratio TG contra = 0.221 ± 0.247; WT contra = 0.456 ± 0.212; ChiSq: 2.469, mean ranks: 5.43/8.83, p = 0.120). To better understand how the lack of mLVs affects the T cell-mediated neuro-immune response, we analyzed both the absolute numbers of CD4 and CD8 subpopulations and their relative frequency. Data analysis shows a reduction of the total number of CD4+ T cells infiltrating the perilesional cortices of TG (median = 106; Q3-Q1 = 156) compared to WT mice (median = 245; Q3-Q1 = 218; ex. coef.: -0.82, p = 0.033 TG ipsi vs. WT ipsi) (**Figure 3A**). No differences were observed in the absolute number of infiltrating CD8+ T cells between the genotypes (**Figure 3B**). Despite no differences in absolute numbers of both CD4 and CD8 populations in the contralateral cortices of injured WT and TG mice, we found a significant reduction in the frequency of CD4+ T cells in transgenic mice (TG contra = 12.04 ± 8.47%; WT contra = 23.59 ± 9.52% of T cells; ChiSq: 3.931, mean ranks: 5.29/9.71, p = 0.042) and a relative increase in the frequency of CD8+ T cells (**Figures 3C, D**), indicating a specific impairment in the CD4-mediated neuro-immune response. As mLVs are involved in the drainage of solutes from the interstitial and cerebro-spinal fluids mainly to the dcLNs (15, 16), we hypothesized that mLVs absence in TG mice can affect the priming of the evoked

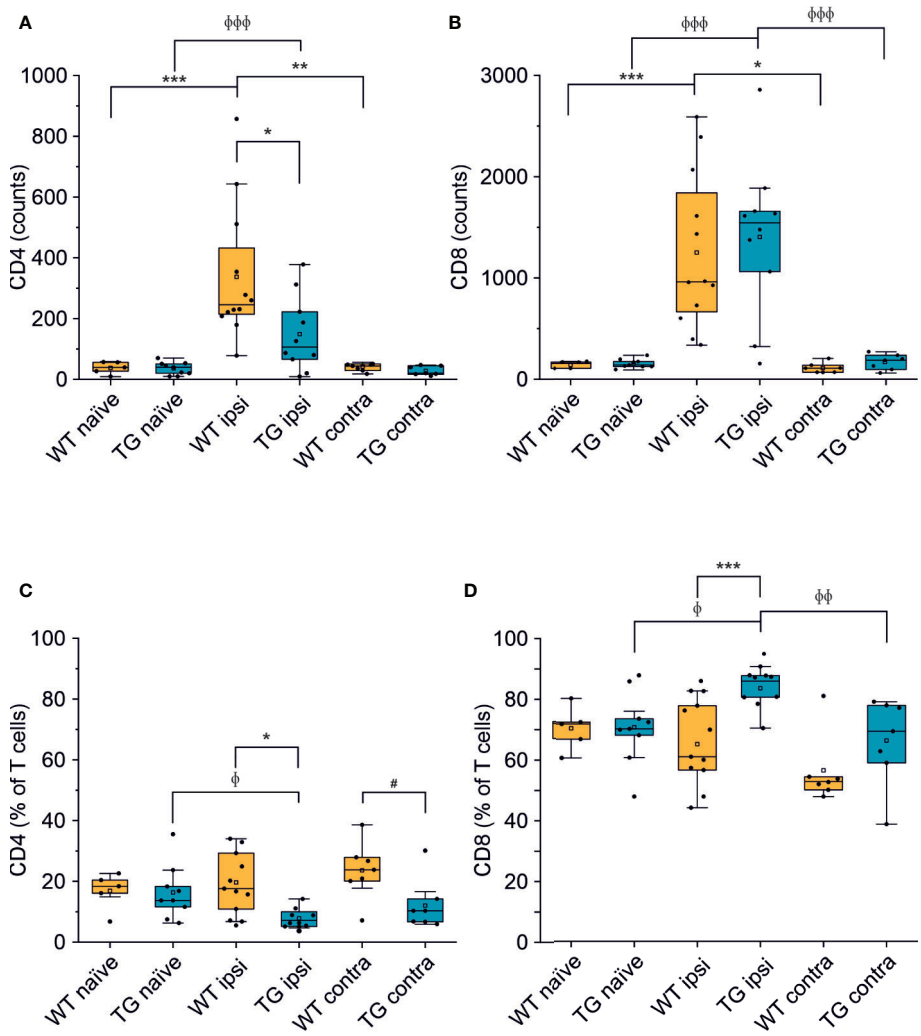


FIGURE 3 | The number of CD4+ but not of CD8+ T cells is reduced in the brain of K14-VEGFR3-Ig mice after TBI. Box plots representing the number and frequency of CD4+ T cells (**A, C**, respectively) and CD8+ T cells (**B, D**, respectively), in the brain of WT and TG mice, as analyzed in the perilesional and contralateral cortices (WT ipsi, n = 12; WT contra, n = 7; TG ipsi, n = 10; TG contra, n = 7), or in intact cortices from naïve mice (WT naïve, n = 5, TG naïve, n = 9). A drastic reduction in the number of CD4+ T cells was found in TG mice after injury. A binomial negative regression or a linear mixed model was applied to assess statistical differences in the counts of CD4+ and CD8+ T cells. The Kruskal Wallis test or the paired samples Wilcoxon signed ranked test was used for the analysis of frequency distribution. *p < 0.05; **p < 0.01 and ***p < 0.001 vs. WT ipsi. $^{\phi}$p < 0.05; $^{\phi\phi}$p < 0.01 and $^{\phi\phi\phi}$p < 0.001 vs. TG ipsi. $^{\#}$p < 0.05 vs. WT contra. In all tests, Bonferroni correction was used to adjust p-values in multiple comparisons.

neuro-immune response. The analysis of the T cell subpopulations in the dcLNs indeed revealed a marked difference between the two genotypes. As expected, we found a significantly lower number of T cells in the dcLNs of the TG-CCI mice (median = 73,542; Q3-Q1 = 21,342) compared to their WT-CCI littermates (median = 220,434; Q3-Q1 = 88,745; p = 0.006). However, TG-CCI mice had a higher frequency of CD4+ T cells (TG CCI = 63.98 ± 5.67%; WT CCI = 51.40 ± 1.93% of T cells; ChiSq: 6.545, mean ranks: 7.50/2.50, p = 0.0017) (**Supplementary Figure 3**). Within the CD4+ T cell subpopulation in the TG mice, cells have predominantly a $CD44^{hi}CD69^{+}$ phenotype, while in the WT mice the predominant population is $CD44^{int}CD69^{-}$ (**Supplementary Figure 3**). No differences were found in the frequency of Tregs.

Different subpopulations of CD8+ and CD4+ T cells exist, with specific and opposing functions: we characterized both the CD8+ and CD4+ subpopulations in the brain for the surface expression of the antigens CD44 (a memory and activation marker) (39, 40) and CD69 (an activation and tissue retention marker) (41). In the perilesional cortex of both WT and TG mice, CD8+ T cells had a predominant $CD44^{hi}CD69+$ phenotype (69.78 ± 22.85% and 72.05 ± 19.95% of CD8+ T cells, in WT ipsi and TG ipsi, respectively) (**Figures 4A, C, D**). In the mouse, the expression of CD69 together with high levels of CD44 define a specific subpopulation of T cells called mature resident memory T cells (T_{RM}) (42–44), which are generated and persist in the tissue at the site of a primary infection (43, 45) and provide a first and powerful line of adaptive cellular defense.

The second-highest expressed CD8+ subpopulation (representing 27.07 ± 26.10% in WT and 25.24 ± 18.85% in TG mice) presented a $CD44^{hi}CD69-$ phenotype, characteristic of effector memory T cells (43). The presence of other CD8+ subpopulations among perilesional infiltrating T cells was negligible. No genotype-related difference was found (**Figures 4C, D** and **Supplementary Table 2**).

Among CD4+ perilesional infiltrating T cells, we found a similar frequency of CD44 and CD69 expressions, with a slight prevalence of $CD44^{hi}CD69+$ over $CD44^{hi}CD69-$ T lymphocytes (**Figures 4B, E, F**) in both genotypes. The overall frequency distribution of the different subpopulations was identical between the two genotypes (**Figures 4E, F** and **Supplementary Table 2**).

K14-VEGFR3-Ig Mice Present a Different Temporal Profile of the T Cell-Mediated Neuroimmune Response After Traumatic Brain Injury

To confirm that the elicited neuro-immune response is specifically affected in the K14-VEGFR3-Ig mice, we analyzed in two different cohort of mice the phenotype of the brain-infiltrating T cells at 3 and 60 dpi. As previously reported (7, 9–11, 46), T cell infiltration peaks at 3 dpi: however, this time frame is not compatible with the priming of the adaptive immune response, and the infiltration of T cells is function of the circulating compartment. At this time point, in our experimental preparations, we did not observe a significant increase in the number of TCRβ+ cells infiltrating the perilesional areas in either of the genotypes (WT ipsi median = 331.5, Q3-Q1 = 409; WT contra median = 99, Q3-Q1 = 53.5; TG ipsi median = 397, Q3-Q1 = 302; and TG contra median = 72, Q3-Q1 = 27) (**Figure 5A**). This could be explained by the fact that, at 3 dpi, T cells mainly enter the brain and accumulate in the area of lesion (removed in our preparation), where the blood-brain barrier (BBB) is damaged. Moreover, analysis of the CD4:CD8 ratio within the infiltrating T cells (**Figure 5B**), did not reveal any difference between WT and TG mice (CD4:CD8 ratio TG ipsi = 1.032 ± 0.323; WT ipsi = 0.964 ± 0.198; ChiSq: 0.5, mean ranks: 4.66/3.50, p = 0.530), thus suggesting that T cells at 3 dpi are recruited independently of the mLVs-dcLNs circuit activation. Next, we characterized the T cell infiltration at 60 dpi chronic time point, to evaluate the progression of the neuro-immune response in the two genotypes. Presence of TCRβ+ T cells in both the genotypes was higher in the perilesional areas (WT ipsi median = 625; Q3-Q1 = 291; TG-ipsi median = 642.5; Q3-Q1 = 497.5), compared to the contralateral cortices (WT-contra median = 227; Q3-Q1 = 77, p = 0.014 vs. WT-ipsi; TG-contra median = 163; Q3-Q1 = 69.5, p = 0.062 vs. TG-ipsi). As observed at 30 dpi, we found a prevalence of CD8+ T cells among the infiltrating lymphocytes, however no genotype or lesion effect was observed from the analysis of CD4:CD8 ratio (CD4:CD8 ratio TG ipsi = 0.220 ± 0.184 WT ipsi = 0.379 ± 0.254; TG contra = 0.218 ± 0.058; WT contra = 0.443 ± 0.162). Analyses of CD4+ and CD8+ subpopulations, revealed fundamental changes in subpopulation frequencies between the two genotypes. Among CD8+ T cells, $CD44^{hi}CD69+$ phenotype was prevalent in WT ipsi, while the $CD44^{neg}CD69+$ phenotype was prevalent in TG ipsi ($CD44^{hi}CD69+$: 60.58 ± 11.26% and 34.75 ± 8.00% in WT ipsi and TG ipsi respectively, p = 0.009; $CD44^{neg}CD69+$: 28.53 ± 14.01% and 53.10 ± 7.27% in WT ipsi and TG ipsi respectively, p = 0.004). These data suggest an activation of CD8-mediated neuro-immune response in the perilesional area of K14-VEGFR3-Ig mice, while cytotoxic cells resident in the injured brain of WT mice conserve a memory phenotype. Within CD4+ population, most of the cells in both the genotypes presented a $CD44^{neg}CD69-$ phenotype. However, in TG mice we observed a tendency towards a frequency increase in the $CD44^{neg}CD69+$ subpopulation (6.44 ± 11.91% and 23.98 ± 10.90% in WT ipsi and TG ipsi respectively, p = 0.052), supporting the hypothesis of a specific activation of the neuro-immune response in the K14-VEGFR3-Ig mice at 60 dpi.

Cortical Lesion is Similar in K14-VEGFR3-Ig Mice and in Their Wild Type Littermates

Analyses of MRI images acquired 21 days after TBI induction revealed a T2 intensity increase in the ipsilateral hemisphere. The increase of T2 intensity was observed in parietal-temporal cortices, mainly involving the somatosensory and visual cortices (**Figure 6A**), expanding in a few cases to the underlying hippocampus (**Supplementary Figures 5C, D**). No significant change of T2 intensity was found between the two genotypes. In the WT CCI group the contusion volume was

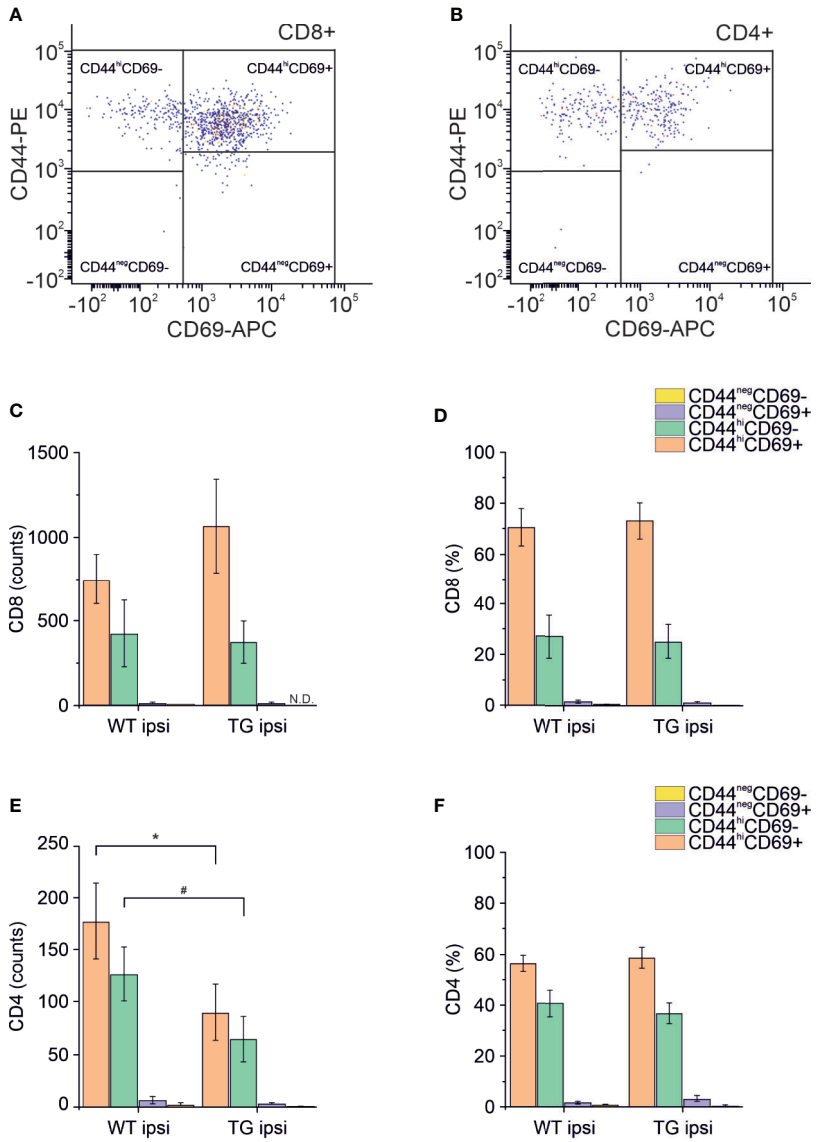


FIGURE 4 | Analysis of CD69 and CD44 T cell activation and memory markers in CD4+ and CD8+ subpopulations. Pseudocolor dot plots **(A, B)** represent gated subpopulations CD69 vs. CD44 of CD8+ and CD4+, respectively. Bargrams in **(C, D)** show respectively the counts and frequencies of CD8+ T cell subpopulations, as analyzed in the perilesional cortices of WT and TG mice. No significant differences in CD8+ subpopulations were found between genotypes. In CD4+ subpopulation, instead, we observed a significant reduction in the counts of $CD44^{hi}CD69+$ and $CD44^{hi}CD69-$ subpopulations **(E)**, in K14-VEGFR3-Ig compared to WT mice. However, no differences were observed in the different subpopulation frequencies **(F)**. Data are presented as median ± SD. A binomial negative regression was applied to assess statistical differences in the counts of total T cells between WT ipsi and TG ipsi. The Kruskal Wallis test was used for the analysis of frequency distribution. $^{\#}p < 0.05$; $^{*}p < 0.05$ vs. WT ipsi.

4.53 ± 1.33%, and 4.09 ± 2.00% in the TG CCI animals (ChiSq: 0.579, mean ranks: 8.71/10.75, p = 0.463) (**Figure 6B**). Relative brain atrophy was 2.42 ± 1.09% in WT CCI mice and 2.00 ± 1.26% in TG CCI mice (ChiSq: 1.400, mean ranks: 8.00/11.17, p = 0.248) (**Figure 6C**). Correlation between contusion volume and relative brain swelling was compared in transformed data analyzed by linear regression. When considering the individual values independent of the genotype, the contusion volume values significantly correlated with the values of relative brain atrophy (r = 0.57; p = 0.023) (**Figure 6D**). No significant correlation was found between the contusion volume and the mean value of the brain atrophy in both the TG CCI group (r = 0.74; p = 0.064), and in the WT CCI mice (r = 0.37; p = 0.331). No differences in lesion progression between WT-CCI and TG-CCI mice have been found from the analysis of T2 MR Images acquired at 3 and 14 days post-injury (**Supplementary Figures 5A, B**).

It must be noted that we have identified the lesion size as the hyper-intense signal in the cortical area observed in the T2

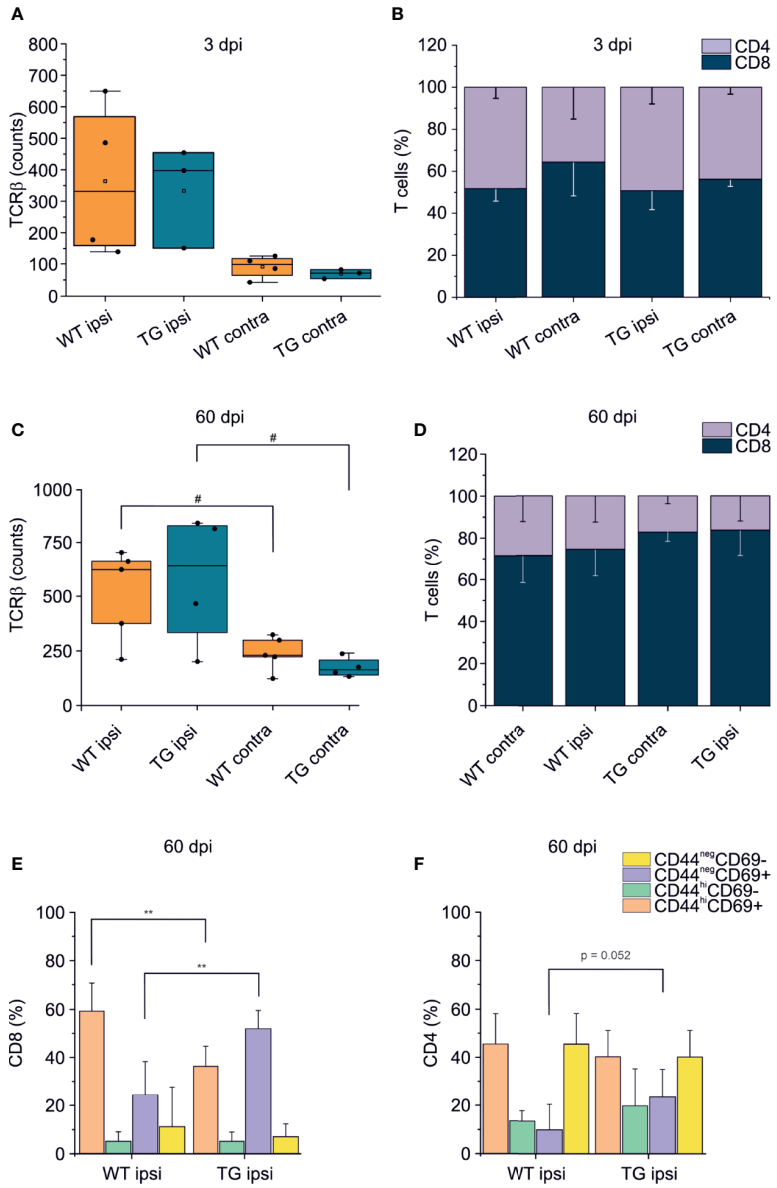


FIGURE 5 | T cell immune response after TBI progress differently in K14-VEGFR3-Ig and WT littermate mice. Panels **(A, B)** represent the number and frequency of TCRβ+ T cells **(A)** and the CD4/CD8 ratio **(B)** in the brain of WT and TG mice, as analyzed in the perilesional and contralateral cortices 3 days post injury (WT ipsi, n = 4; WT contra, n = 4; TG ipsi, n = 3; TG contra, n = 3). No differences between the genotypes have been observed. **(C–F)** Analysis of T cells infiltration in the brain of K14-VEGFR3-Ig and WT littermate mice 60 days post-injury (WT ipsi, n = 5; WT contra, n = 5; TG ipsi, n = 4; TG contra, n = 4). Box plot represents the number of infiltrating T cells, defined by expression of TCRβ **(C)** and stacked bargram represents the percentage of CD4+ and CD8+ T cells **(D)** in the perilesional areas (ipsi) and correspondent contralateral areas (contra) of WT and TG mice. Bargrams in **(C, D)** show respectively the frequencies of CD8+ and CD4+ T cell subpopulations, as analyzed in the perilesional cortices of WT and TG mice. In CD8+ subpopulation we observed a significant reduction in the frequency of the $CD44^{hi}CD69+$ subpopulation in K14-VEGFR3-Ig compared to WT mice, which corresponded to the increase in the frequency of $CD44^{neg}CD69+$ phenotype. In CD4+ subpopulation, instead, we did not observed differences in distribution between the two genotypes. Data are presented as median ± SD. A binomial negative regression or a linear mixed model was applied to assess statistical differences in the counts of TCRβ + T cells. The Kruskal Wallis test was used for the analysis of frequency distribution. **p < 0.01 vs. WT ipsi. #p < 0.05 vs. respective contra. In all tests, Bonferroni correction was used to adjust p-values in multiple comparisons.

weighted images. Our analysis, albeit clinically relevant, suffers from a lack of spatial definition and is affected mostly by the formation of the cyst at the site of injury (27, 47). Therefore, subtle although significant differences in the lesion size can be underestimated. However, the analysis of MAP-2 staining in the brain of the WT CCI and TG CCI animals confirmed the MRI results and did not show any genotype-related differences (**Figure 6E**).

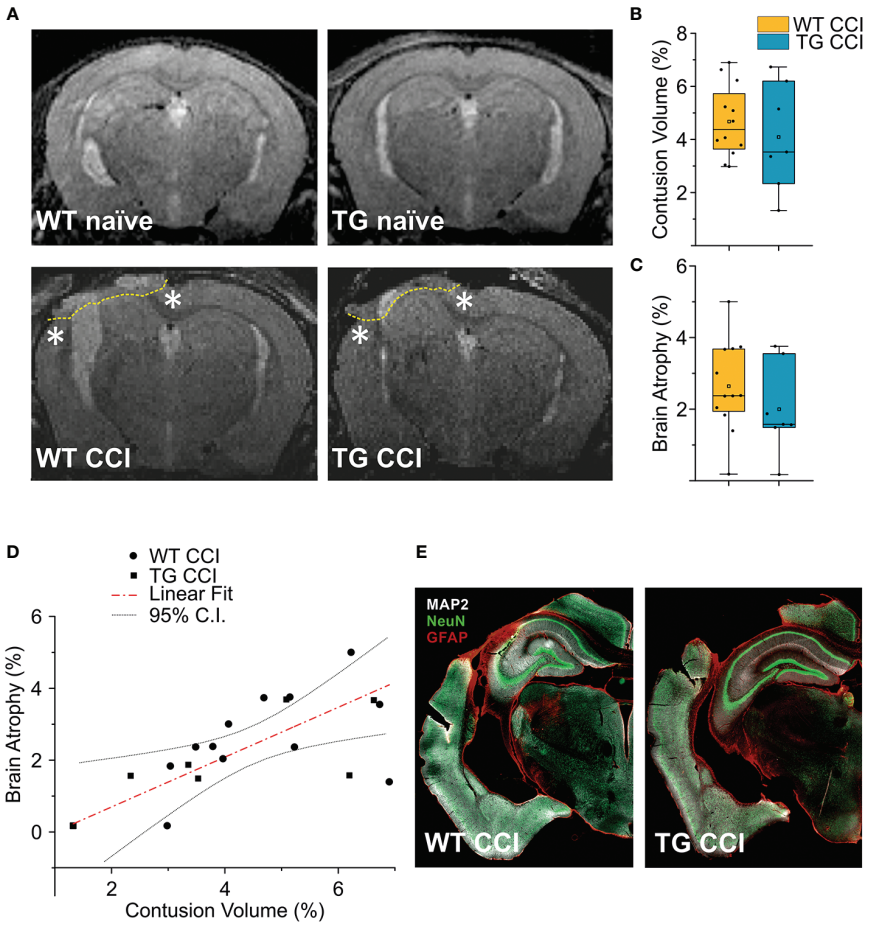


FIGURE 6 | TBI-induced lesions does not differ between the two genotypes, as inferred by the analysis of MRI at 21 dpi. **(A)** Representative MR images of WT naïve, WT CCI, TG naïve and TG CCI brains. Perilesional cortices in WT CCI and TG CCI brains are marked with stars. Box plots in **(B, C)** illustrate the genotype effect on the percentage of contusion volume and of brain atrophy, respectively, over the volume of the hemisphere ipsilateral to the lesion. No significant differences were observed between TG K14-VEGFR3-Ig and WT mice. For the definitions of the contusion volume and of brain atrophy see the main text. **(D)** When considering the contusion volume and the brain atrophy independently from the genotype, we found a direct correlation between the two parameters. **(E)** Representative images of WT CCI and TG CCI brains stained for MAP2, NeuN, and GFAP at 30 dpi. No differences in neuronal damage or in neuroinflammation were visible between the two genotypes, supporting the MRI *in vivo* data. The Kruskal Wallis test was used for the analysis of infarct volume and of tissue loss between the two genotypes. CI: 95% confidence interval. For box plot explanation, refer to the legend of **Figure 2**.

K14-VEGFR3-Ig Mice Present a Peripheral Lymphopenia, Which is Exacerbated After Traumatic Brain Injury

Alterations of systemic immunity are frequent in TBI patients. We analyzed the levels and the frequency of different T cell subpopulations in the spleen of WT and TG mice, one month after TBI induction. As previously described (48), K14-VEGFR3-Ig mice show a moderate lymphopenia compared to littermate WT mice (percentage of T cells over live cells in WT naïve: 37.26 ± 7.67%; vs. TG naïve: 19.69 ± 4.96%; ChiSq: 14.746, mean ranks: 5.00/15.50, p = 1e-04) (**Figure 7A**). In TG mice, but not in WT mice, we found a significant reduction in the total T cell frequency after TBI (WT CCI: 33.68 ± 6.99%; TG CCI: 14.23 ± 2.87% of live cells; ChiSq: 7.695, mean ranks: 7.18/14.55, p = 0.003 TG CCI vs. TG naïve) (**Figure 7A**), confirming that TG mice present an impaired immune response, which relates to the alterations in the lymphatic system. Contrary to what was observed in the brain, the systemic lymphopenia in the K14-VEGFR3-Ig genotype corresponds to a relative frequency reduction in peripheral CD8+ T cells (TG naïve = 25.75 ± 3.61%; WT naïve = 42.70 ± 4.17% of T cells; ChiSq: 14.727, mean ranks: 5.00/15.50, p = 1e-04) (**Figure 7B**). Analysis of the activation markers show a different expression in both CD4+ (**Figures 7C, E**) and CD8+ (**Figures 7D, F**) subpopulations between WT and TG mice, which is trauma independent. Both TG naïve and TG CCI mice, indeed, showed an increased frequency of memory T cells (CD4+CD44^{hi}CD69+, CD4+CD44^{hi}CD69- and CD8+CD44^{hi}CD69+, CD8+ CD44^{hi}CD69-; for statistical analysis, see **Supplementary Table 3**).

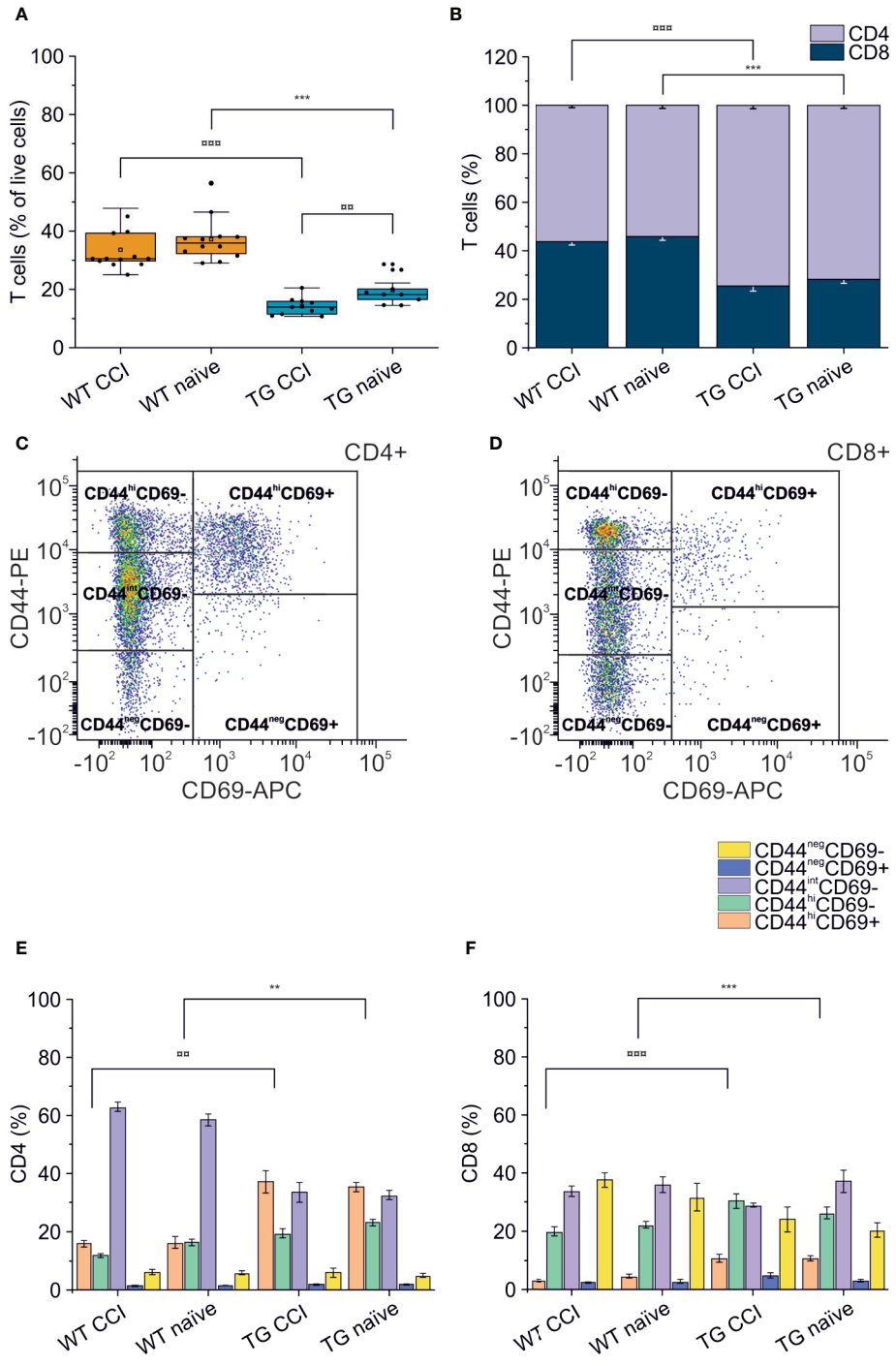


FIGURE 7 | Peripheral immune response in the spleen. The percentages of T cells in the spleen of WT naïve and CCI mice and of TG naïve and CCI mice are presented in the box plot in panel **(A)**. Stacked bargrams in **(B)** represent the relative percentages of CD4 and CD8 in T cell population, in WT and K14-VEGFR3-Ig mice. K14-VEGFR3-Ig mice present a drastic reduction of T cells compared to WT littermates, due to a decrease in CD8+ T cell frequency. **(C, D)** Representative pseudocolor dot plots and gating strategies for CD4+ and CD8+ T cell subpopulation analysis, respectively. Bargrams in **(E, F)** show respectively the frequencies of CD4+ and CD8+ T cell subpopulations, as analyzed in WT and TG mice. Significant differences in the frequencies of both CD4+ and CD8+ subpopulations have been observed. The Kruskal Wallis test or the paired samples Wilcoxon signed ranked test was used for the analysis of frequency distribution. ¤¤$p < 0.01$ and ¤¤¤$p < 0.001$ vs. TG CCI. **$p < 0.01$ and ***$p < 0.001$ vs. WT naïve. In all tests, Bonferroni correction was used to adjust p-values in multiple comparison. For box plot and stacked bargram explanation, refer to the legend of **Figure 3**.

DISCUSSION

This study analyzes the progression of T cell-mediated neuro-immune response as a result of a single moderate TBI, in a mouse model characterized by a developmental deficiency in the CNS lymphatic system.

Mounting evidence implicates a sustained modulation of T lymphocyte-mediated immune response following TBI, both in patients (49–51) and in animal models of brain injuries (7, 9–11, 46).

A recent publication from Daglas and colleagues characterized for the first time the T cell-mediated immune response in a chronic animal model of TBI, highlighting the role of cytotoxic CD8+ T cells in the progression of TBI pathology (12).

Our data confirm the previous findings, showing a sustained accumulation of CD8+ T lymphocytes, restricted to the non-damaged cortical areas surrounding the lesion and to the underlying corpus callosum, already at 30 dpi (*i.e.*, the early chronic phases after TBI). Moreover, we expand the current knowledge characterizing the phenotype of the accumulating lymphocytes as putative resident memory T cells. Our data suggest a direct in-situ activation of the T cell-mediated immune response, which could play a role in the progression of TBI pathology, as previously indicated (12).

We also found that the congenital lack of the meningeal lymphatic system affects the polarization of the TBI-elicited T cell immune response, and its progression over time. Finally, we found that the adaptive neuro-immune response is prompted even in the absence of a systemic immune reaction.

Specifically, our findings suggest that at early chronic time points after TBI: 1) the immune response in the brain is principally mediated by putative T_{RM} CD8+ cells; 2) the CNS lymphatic system modulates the specific neuro-immune response; 3) the systemic T lymphocyte response does not correlate with the neuro-immunological state of the brain.

Brain trauma results in two phases of tissue injury. The primary injury which is a direct result of the mechanical impact to the brain, is characterized by the activation of the innate immune response and the release of excitotoxic agents. During this acute phase, a massive and dysregulated brain-infiltration of T cells has been reported (52, 53). This infiltration is presumably confined to the area of the lesion, since we observed a limited number of infiltrating T cells in the perilesional non-injured areas, 3 days after TBI induction (**Figure 5A**). A secondary tissue damage, resulting in a diffuse and long-lasting injury, usually develops after months/years from the primary injury (54–56). This is characterized by additional neurodegeneration developing independently from the mechanical trauma and by the formation of a fibrotic scar tissue in the injured area (57) (**Figure 6E**). It has been recently suggested that the development of secondary injuries is sustained by activated memory CD8+ T cells (12). In CCI mouse model (similar to the one used in this study), the authors observed that the modulation of the cytotoxic lymphocytes resulted in the reduction of the lesion size and in the improvement of the neurological outcomes analyzed 32 weeks after injury.

In similar experimental conditions, we observed that CD8+ T lymphocytes with a $CD44^{hi}CD69+$ phenotype are already present in the perilesional areas (but not in the correspondent contralateral cortices) one month after TBI. Since CD69 is an early marker of T cell activation (41) and inhibits tissue egression (45), our data suggest a localized activation of the resident memory CD8+ subpopulation (42–44) restricted to the areas surrounding the primary lesion. In the case of TBI, $CD44^{hi}CD69+$ T_{RM} cells may represent the population designated to defend the non-injured brain from possible infective agents penetrating through the lesion. However, within the chronic neuro-inflammatory environment observed in the perilesional areas (**Figure 6E**), we propose that T_{RM} can activate in a dysregulated way. Indeed, our data indicate that, 2 months after TBI, CD8+ T cells present around the lesion shift towards a $CD44^{neg}CD69+$ phenotype, typical of functional differentiated tissue-resident T cells. This may contribute to the cytotoxic immune response, which characterizes the chronic phases of TBI pathology. Our hypothesis is supported by the data reported by Daglas and colleagues (12), indicating that brain infiltrating CD8+ T cells express and release effector cytokines (Granzyme B and IFNγ). Further studies are required to determine if this adaptive response is antigen specific, and if secondary lesions are the result of an autoimmune-like sequelae of events.

Neuro-immune responses are mainly elicited in the dc- and scLNs (18–20, 58, 59), which are the main receivers of the mLVs. Therefore, the meningeal lymphatics represent an integrated component in the neuro-immune response (15), and we hypothesize that mLV functional impairment can affect the priming of the T cell-mediated neuro-immune response following TBI.

We addressed this hypothesis by inducing TBI in a transgenic mouse, modelling a congenital lymphedema. K14-VEGFR3-Ig mice, expressing soluble VEGFR-3-Ig (21), present alterations in the development of the lymphatic system, resulting in defective growth of mLVs and in sclerotic dcLNs (16, 17). This phenotype has been confirmed in our experimental animals.

We found that the neuro-immune response in the K14-VEGFR3-Ig mice significantly differs from the response observed in WT mice after TBI, suggesting that a developmental defect in the CNS lymphatic system directly affects the CNS regional immune regulation and modulates its chronic activation in the TBI pathology. This hypothesis is supported by the observation that the initial BBB damage-associated T cell infiltration in the perilesional areas was similar in the two genotypes (**Figures 5A, B**), whereas at 30 dpi (and partially at 60 dpi) we found a marked decrease in the CD4+ T cell frequency in the TG mice (**Figures 2A, B** and **Figures 5C, D**). This results in the polarization of the neuro-immune response towards CD8+ cytotoxicity, possibly aggravating TBI outcomes as recently suggested (12). Moreover, at the most chronic time point analyzed in this study (60 dpi), we also observed a different evolution of the CD8-mediated response, with T cells from the brain of TG mice shifted towards a $CD44^{neg}CD69+$ phenotype, and the one from WT littermates still presenting mainly a T_{RM} phenotype (**Figure 5E**). It is important to note, however, that K14-VEGFR3-Ig mice have a compromised

peripheral immune response (22, 48, 60, 61), which could affect the local immune response observed in the brain.

Indeed, in chronic TBI animals, the analysis of the T cell subpopulation in the CNS-draining dcLNs also showed a marked difference between the two genotypes. CD4+CD44^{hi}CD69^{+} T cells were the predominant subpopulation in TG mice, and CD4+ CD44^{int}CD69^{neg} T cells were predominant in WT mice (**Supplementary Figures 3D, E**). It has been suggested that CD4+CD44^{int} T cells could represent the fraction of central memory T helper cells expressing IFN-γ, while CD4+CD44^{hi} would preferably be effector memory cells with a Th17 phenotype (62, 63). A polarized Th1/Th17 response has been reported in CNS autoimmune diseases (64) and can enhance the cytotoxicity of CD8+ T cells (7, 12). This would support the differences in the neuro-immune response observed in our TG animals and partially explain the direct correlation we found in these mice between the frequency of CD4+ T cells and the brain atrophy (**Supplementary Figure 3**). However, the panel of antibodies we used for T cell characterization does not allow us to distinguish between the different CD4+ T helper populations (*i.e.*, Th1, Th2, or Th17) without speculation.

Our data suggest that the developmental impairment of mLVs observed in K14-VEGFR3-Ig mice is associated with a different modulation of the adaptive neuro-immunity in response to TBI. We here conjecture on the possibility that in both WT and K14-VEGFR3-Ig mice, as the result of trauma, the brain-derived antigens escape directly into the blood, activating a CD8-mediated immune response in secondary lymphoid organs, with T cells freely accessing the lesion site due to the damage in the BBB. This results in a similar activation of the primary adaptive immune response which eventually generates the brain resident memory T cells. In WT mice, however, our hypothesis is that antigens are partially drained through the mLVs to the dcLNs, eliciting a Th2-mediated response as previously proposed (18, 20). In K14-VEGFR3-Ig mice, where the functional mLVs-dcLNs connection is absent, this specific response however is restrained, as suggested by our data.

Other mechanisms linked to mLV dysfunction can contribute to the modulation of the neuro-immune response. For instance, lymphatic vessels play a direct role in the maturation of T cells (65, 66), and dysfunction of the lymphatics leads to the persistence of immune cells and mediators in tissues, resulting in a chronic inflammation and tissue damage (67). Moreover, recent papers reported that the VEGFR-3 signaling, promoting lymphangiogenesis, is also important to both initiate the acute innate and adaptive immune responses and to regulate the chronic T cell-mediated response (by changing the Treg/Th2 balance), suggesting an immunomodulatory role for this signaling (68, 69).

It is conceivable, therefore, that the inhibition of the VEGFR-3 signaling in K14-VEGFR3-Ig TG mice, and their congenital lack of mLVs, can affect both the type of the elicited neuro-immune response and its progression.

Interestingly, in a recent paper it has been demonstrated that TBI leads to the temporary impairment in meningeal lymphatic drainage, by increasing intracranial pressure (70). These data suggest that, independently from the pre-existing mLV deficit, drainage of the antigens to the dcLNs should be inhibited during the acute phases following brain trauma. In addition, the same authors demonstrated that prior lymphatic defects are related to the increase in the TBI-induced innate and adaptive immune responses (enrichment gene analysis), and to a more pronounced cognitive deficit, when acutely tested after brain injury (3 dpi) (70). However, in their work Bolte and colleagues used the photosensitizer verteporfin to induce the photodynamic ablation of mLVs, a treatment well known to induce the release of free radicals and the increase of local inflammation after verteporfin activation. Therefore, although these recent observations, together with our data, suggest that pre-existing mLV conditions can promote the neuro-immune response and worsen TBI pathology, more unbiased studies need to be provided to confirm this hypothesis.

Our data, indicating a role of T_{RM} cells in the TBI pathology, could also have important clinical implications. TBI patients generally present a delayed secondary immunodeficiency (CNS injury-induced immunodepression, CIDS) (71, 72), which is accompanied by an increased susceptibility to systemic infections and is associated with declining neurological outcome and increased mortality.

Analysis of our data suggest that neuro-immune reaction can be elicited in the CNS even in the presence of a systemic congenital lymphopenia (as observed in K14-VEGFR3-Ig mice), excluding a correlation between the extent of brain infiltration and the level of T cells in the periphery (**Supplementary Figure 4C**). This observation suggests that patients with CIDS could at the same time present a sustained adaptive immune response localized in the brain. Immunomodulatory therapies directly targeting the brain-resident memory T cells could benefit TBI patients without affecting their already compromised systemic immune system.

Therapeutic approaches aimed at downregulating the adaptive immune response after TBI have been tested before (73) with no improvement on the neurological outcome, leading to the hypothesis that the adaptive immune response after brain injuries can have a beneficial activity (74, 75). However, it is important to note that these studies focused on the manipulation of the early wave of T cell infiltration after TBI. Our findings, together with recently published data, indicate that the chronic immune response is the target for the development of specific therapies for the treatment of TBI patients. This includes modulating the progression of the secondary injuries and opening the way to new studies in this direction.

LIMITATION OF THE STUDY

This work represents a proof of concept for the involvement of adaptive neuro-immunity in TBI pathology and for the role of mLVs in modulating this response.

We are aware that this study presents several limitations and further studies are needed to understand how mLVs regulates the kinetics of activation and brain recruitment of CD8+ T cells after

TBI, and the specific role of these cells in the progression of the pathology. A major limitation stems from the use of K14-VEGFR3-Ig mice with a congenital and global deficiency in the mLVs. This results in a compromised peripheral immune response, as previously demonstrated (22, 48, 60, 61) and confirmed by our spleen data. In their paper, however, Thomas and colleagues reported a delayed but robust CD8-mediated response to peripheral immunization and impaired tolerance. In a similar fashion, we have found an increase in the CD8+ T cell response to putative brain-derived antigens. These data confirm the contribution of lymphatic vessels in the modulation of the adaptive immune response and support the hypothesis that the elicited cytotoxic response can escape the intrinsic brain tolerance. Nevertheless, this hypothesis needs to be confirmed in different models that would study the effects of local partial depletion of the mLVs on the activation of the neuro-immune response (e.g., ligation of the dcLNs at the moment of brain injury).

Another limitation of our study is the lack of difference in lesion size between K14-VEGFR3-Ig mice and their WT littermates despite the increase in the number of cytotoxic T cells. As discussed previously, this could be due to limitations in our analytical approach. However, it is also possible that although triggered by cytotoxic T cells, secondary neurodegeneration and associated behavioral correlates may appear at a later time point than the one analyzed in this study. Specific analyses should be conducted in the K14-VEGFR3-Ig mice (and other models of meningeal lymphatic depletion) to assess the long-term effects of mLV deficits on the progression of TBI pathology.

Finally, our analyses focused on TCRb+ T cells, which represent the main population of T cells responsible for the adaptive immune response. Other immune cells (not analyzed in our study) could play an important role in TBI pathology, representing a possible target for future immunomodulatory strategies. Further studies are needed to fully characterize the contribution of the humoral and cellular neuro-immune response in TBI pathology.

CONCLUSIONS

Our study investigated the phenotype of T lymphocytes infiltrating and persisting in the brain after TBI, pointing to the activation of the CD8+ resident memory T cells in the early chronic response. Our findings also support the importance of mLVs and dcLNs in maintaining brain immuno tolerance. We, therefore, propose that the modulation of the neuro-immune response *via* the CNS-lymphatic system, or by directly targeting the brain-resident memory T cells, could offer therapeutic strategies for the treatment of TBI patients.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Ethics Committee of the Provincial Government of Southern Finland.

AUTHOR CONTRIBUTIONS

SW contributed to the methodology, investigation, validation, data curation, writing, reviewing, and editing. AV contributed to the investigation, data curation, and formal analysis. MV contributed to the investigation, data curation, and formal analysis. BG provided the software and contributed to the formal analysis. MK contributed to the investigation, writing, reviewing, and editing, ER conducted the formal analysis. SA provided the resources and wrote, reviewed, and edited the manuscript. JK supervised the study and acquired the funding. FN conceptualized the study, contributed to the methodology, validated the study, and contributed to the writing, supervision, and funding acquisition. All authors contributed to the article and approved the submitted version.

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This manuscript has been released as a pre-print at BioRxiv (76).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.559810/full#supplementary-material

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frontiers in Immunology

REVIEW
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The Immune System's Role in the Consequences of Mild Traumatic Brain Injury (Concussion)

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Mild traumatic brain injury (mild TBI), often referred to as concussion, is the most common form of TBI and affects millions of people each year. A history of mild TBI increases the risk of developing emotional and neurocognitive disorders later in life that can impact on day to day living. These include anxiety and depression, as well as neurodegenerative conditions such as chronic traumatic encephalopathy (CTE) and Alzheimer's disease (AD). Actions of brain resident or peripherally recruited immune cells are proposed to be key regulators across these diseases and mood disorders. Here, we will assess the impact of mild TBI on brain and patient health, and evaluate the recent evidence for immune cell involvement in its pathogenesis.

Keywords: concussion, neuroimmunology, microglia, neurodegenenerative diseases, inflammation, mild TBI

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MILD TRAUMATIC BRAIN INJURY

Traumatic brain injury (TBI) is a term used to include a spectrum of insults resulting from mechanical injury to the brain. TBI includes injuries that range from severe, with open skull injuries and major parenchymal disruption, to the mildest form of TBI, often termed concussion. Although widely used in everyday language, the term concussion is now less commonly used in medical and scientific terminology, as it lacks diagnostic precision and does not refer to underlying pathological processes (1, 2). Therefore, mild TBI is the preferred term and will be used throughout this review (1, 2). It is estimated that TBI affects 69 million individuals each year world-wide, with the vast majority of cases being mild TBI (3, 4). The main causes of mild TBI are motor accidents, falls, assaults, active-duty of soldiers, and domestic violence (5, 6). Recently, greater attention has been given to this condition due to the high prevalence of mild TBI among young athletes in relation to their involvement in collision sports such as American Football, soccer, and rugby (7).

Mild TBI is a physiological disruption of brain function and occurs due to mechanical distortion of brain tissue, most commonly from a blow to the head, but can also be caused by a blast injury frequently seen in soldiers serving in a war zone (7, 8). Rapid rotational velocity/acceleration (inertial loading) is thought to be a key component of injury (9–11). The underlying pathophysiology of the injury remains poorly understood, as availability of human post-mortem brain tissue for examination from this typically non-lethal injury is limited (12). Although referred to as "mild," individuals can still experience a variety of physical, emotional and cognitive problems, including sleep disturbance, increased anxiety, and depression (4, 7, 13, 14).

Diagnosing mild TBI and its severity is usually based on the loss of consciousness duration (<30 min), the Glasgow coma scale score (13–15), post-traumatic amnesia duration (momentarily to <24 h), and a lack of intracerebral/subdural/epidural hematoma, cerebral, or hemorrhagic contusion, penetrating TBI (dura penetrated), subarachnoid hemorrhage or brainstem injury (2) (**Figure 1**). Mild TBI is still a heterogeneous insult, and there can be major variation in the likelihood of significant neuropathology and varying symptoms including blurred vision, confusion, dizziness, focal neurological symptoms, headache, and nausea (2, 7, 8). Whilst most patients recover and return to their normal self, the clinical outcome of concussion is hard to predict. This is because of the heterogeneity of initial trauma, the inability to quantify disease severity and the likely initiation of complex pathogenic pathways (15). Even though men are at greater risk of mild TBI due to greater participation in high-risk activities, studies have shown that females are at greater risk of poor outcomes (16–19) and further research of both sexes is needed to characterize the nature of sex-dependent injury and recovery (20). In addition, pre-existing health conditions, age, genetic background (21), and alcohol or substance abuse also influences recovery and leads to differences in clinical outcome between patients (22).

The following review will focus solely on consequences of mild TBI in adults, that would be commonly be referred to as concussion. There is a vast and important literature on more severe TBI that includes evidence of hemorrhage and parenchymal injury and these injuries are defined as moderate or severe TBI. Clinically, moderate or severe TBI is diagnosed by neuroimaging and preclinical modeling of these injuries is much more common than mild TBI, due to the production of frank and measurable tissue damage. As a result, there is a large literature on neuroinflammatory and immune mechanisms that drive both injury and repair in these insults (23–25). Much less is known about the pathology of mild TBI with no overt contusion or hemorrhage in the pathology, and here, a different immune response is likely to occur. The current review will explicitly refer to evidence of the immune response after human mild TBI, in animal models with high translational relevance to mild TBI (without compromising the skull and no evidence of hemorrhage) and studies that investigate patients with a history of head injury through sport.

EVIDENCE FOR MILD TBI AS A RISK FACTOR FOR LONG-TERM PROBLEMS

Mild TBI is now recognized as a major public health concern as clinicians and researchers are becoming more aware of the dangers and potential long-term consequences associated with this type of head injury (26). In most mild TBI cases, acutely reported symptoms resolve within 3 months; however, a small proportion of patients continue to suffer life disrupting symptoms (27–29). A range of factors, not necessarily directly reflecting injury severity, are associated with poor outcome following mild TBI, including previous neurological or psychiatric problems and whether the patient had suffered a previous head injury (27, 28). Indeed, patients with a history of mild TBI can experience changes in emotions or behavior, often expressed by increased anxiety and depressive like behaviors (2, 4, 7, 13, 14). Most studies investigating multiple head injuries over a sustained period are derived from participants of contact sports. These patients may differ greatly from those who suffer a mild TBI as a one-off event, not only in the nature of the head injury but also their lifestyle and pre-morbid traits (30). Currently, there is major interest in mild TBI/concussion due to its prevalence in sports such as the National Football League (NFL), rugby, and soccer, where the risk of head injury is high. Single concussive events in these sports can result in the same myriad of symptoms as a one-off mild TBI in the general population, and may trigger that same initial pathological response; however, it is the accumulation of injuries and their long-term effect on mood and neurodegenerative outcomes that is often assessed in these athletes.

Effects of Repeated Mild TBI in Contact Sports

In contact sports, diagnosed mild TBIs/concussions and even head impacts that are frequent but do not cause noticeable immediate injury, such as heading a soccer ball, are now being investigated as risk factors for poor long-term brain health (31). In a population of retired rugby players, the prevalence of major depressive disorder was significantly higher compared to other retired sportsmen (7). Another study investigated professional NFL players and found a link between recurrent concussion and diagnosis of lifetime depression and suggested that the prevalence of depression increases with the number of past mild TBIs (32). Indeed, retired players that reported either one to two, or three or more previous concussions were 1.5 and three times more likely to be diagnosed with depression, respectively, compared to retired players with no history of mild TBIs (32). Regardless of the type of contact sport, diagnostic test scores for major depressive disorder increases with the number of mild TBIs (7).

In addition to emotional disturbances, mTBI is associated with a risk of developing a number of neurodegenerative conditions (33). A series of studies retrospectively investigating a cohort study of former professional footballers [Football's Influence on Lifelong health and Dementia risk (FIELD)] investigated the link between at dementia pathology, mortality and mental health and suicide in ex footballers (soccer players) (34). Mortality from neurodegenerative disease was higher and mortality from other common diseases lower among retired professional soccer players than among matched controls (35). Surprisingly, in these cohorts, hospital admissions for common mental health disorders were lower than population controls, with no difference in suicide, despite evidence of neurodegeneration (36). Outside of professional athletes, a population-based administrative health cohort study, in more than 47,000 cases of mild TBI showed mild TBI was associated with an increased risk of diagnosis of attention-deficit hyperactivity disorder, mood and anxiety disorders, dementia and Parkinson's disease later in life (37).

The majority of evidence suggests that mild TBI can be detrimental to mental health, but also carries increased risk of developing epilepsy and neurodegenerative disorders, such as

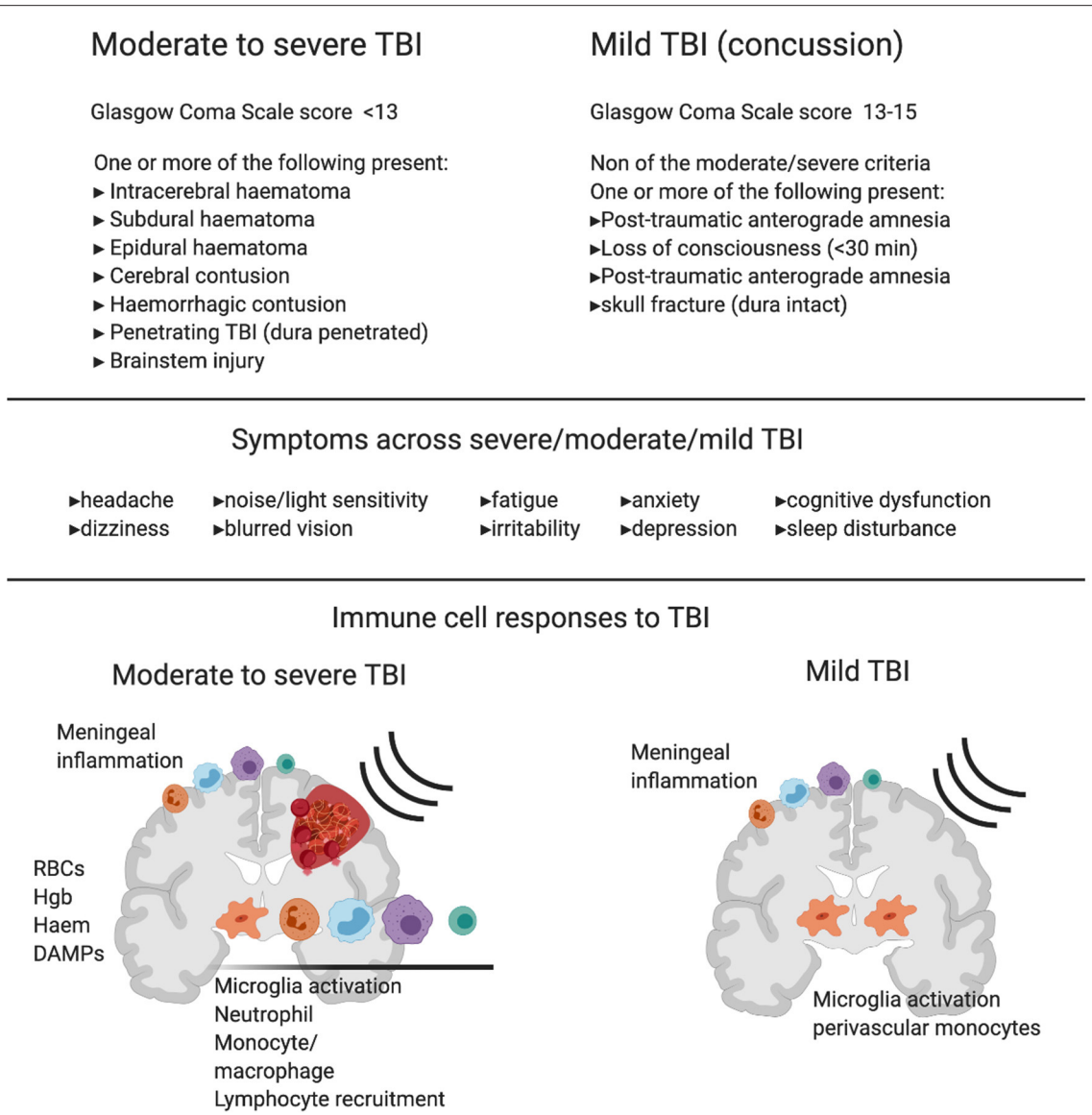

FIGURE 1 | Diagnostic criteria, symptoms and immune cell involvement in moderate to severe traumatic Brain Injury (TBI) in comparison with mild TBI. Commonly used diagnostic criteria in moderate to severe TBI compared to mild TBI shows the major clinical difference between the two reflects hemorrhage or clear contusion in the brain. Symptoms are shared across mild, moderate and severe TBI with increasing likelihood of symptom occurrence and severity with increasing injury. Schematics represent immune response in moderate to severe TBI (left) and mild TBI (right). In moderate to severe TBI in humans and animal models, there is clear evidence for resident microglia activation and recruitment of macrophages, dendritic cells, neutrophils, B cells and T cells, and meningeal inflammation. In addition to active recruitment mechanisms, peripheral immune cells can infiltrate with frank hemorrhage alongside red blood cells (RBCs) and the release of hemoglobin (Hgb), Haem, and other damage associated molecular patterns (DAMPs), which are one set of initiators of the immune response. In contrast, in mild TBI there is little evidence of infiltrating immune cells to the brain tissue in humans or animal models that do not produce hemorrhage or skull opening. In mild TBI, there is evidence of meningeal inflammation, microglial activation, and some monocyte/macrophage recruitment to the cerebrovasculature.

Alzheimer's disease, Parkinson's disease, and chronic traumatic encephalopathy (CTE) (2). Significant neurodegeneration observed in retired athletes has been linked to repeated mild TBI at a younger age and mortality from neurodegenerative disease is significantly higher among former professional soccer players than in matched population controls (35). Within this group, Alzheimer's disease as the primary or a contributory cause of death was responsible for the largest increase of deaths (35). In a separate study, there was increased risk and early onset of ALS in professional players from Italian soccer teams (38).

Post-mortem studies on former contact sports athletes show a high prevalence of CTE; a progressive neurodegeneration associated with repetitive head trauma (39, 40). Interestingly, neuropathological severity of CTE seemed to increase in

accordance with the level of play and almost all cases had behavioral or mood symptoms or both, cognitive symptoms or signs of dementia (39). Post-mortem brains from U.S. military veterans that have been exposed to blast exposure and/or concussive injury display CTE neuropathology that is similar to the pathology observed in former athletes (41).

These studies add to the growing evidence to suggest that a history of mild TBI is a risk factor for the development of pathological neurodegeneration. They have also brought much needed attention to the dangers of head injury, in general. Research is now focused on understanding the underlying pathology of both single and accumulated mild TBIs, and what can be learned from each individual instance to prevent long-term problems.

Understanding Distinct or Overlapping Mechanisms of a Single, Repeated, or Life-Long History of Mild TBI

The *Lancet* Commission on dementia prevention recently added TBI as potentially modifiable risk factor for dementia (26). In this important document, a combination of studies relating to severe TBI, those investigating concussion, or a career in professional contact sport, are cited as why *traumatic brain injury* is considered a risk factor for dementia and neurodegenerative disease (26, 33). As detailed above, severe TBI can be quite different to mild TBI. However, the role of neuroinflammation, propagated by the immune system, is a likely modifiable regulator in both. Even when focusing on mild TBI research alone, there are still many challenges to assessing the role of the immune system. Complicating factors include: heterogeneous pathology and symptoms (2), diffuse injury across brain regions (10, 42) and the overlapping research conclusions between one-off mild TBI and multiple injuries sustained by professional sport participation. However, the investigation of the immune response to discrete injuries will undoubtedly lead to increased understanding of the dangerous cumulative effects of multiple mild TBIs. Indeed, much of our understanding of the mechanisms of mild TBI, and the role of the immune system, is derived from animal models of injury. A combination of clinical and preclinical studies in the acute setting can provide insight into both mild TBI in the general population and those accumulated in professional sport.

The following sections will outline what is known about the immune system's response to CNS injury in general, and why aspects of the immune system have become considered drivers of neurodegenerative disease, of which head injury is now considered a risk factor. This will provide context for the review of the current literature for immune involvement in the pathology of mild TBI.

POSITIONING OF THE IMMUNE SYSTEM WITHIN THE CNS

Immune cells are present throughout the adult CNS (43). Microglia are a type of tissue resident macrophage and are the major immune cell type (44). Microglial cell bodies and their processes cover every cubic micrometer of the brain during constant surveillance activities (45, 46). Recent work shows that, of the total number of immune cells in the brain, ∼80% are microglia, with the remainder comprised of barrier-associated macrophages and cell types more traditionally associated with the periphery such as neutrophils and T cells (44, 47–49). Microglia are highly plastic and defend the brain against external challenges. Pattern recognition receptors (PRRs) are scattered along their membrane, by which they can recognize pathogen-associated molecular patterns (PAMPs) and host-derived danger-associated molecular patterns (DAMPs), making microglia equipped with the tools to evoke a rapid, fine-tuned inflammatory response to immunological challenges (50). Microglia phenotype and morphology are determined by their local environment and several concepts relating to their function, such as "homeostatic," "primed," "trained," or "tolerant" microglia, have emerged from experimental models (51). Microglial priming is defined as a prolonged and exaggerated immune response resulting from an acute inflammatory event in an ongoing inflammatory environment (52). Innate immune memory is associated to cell reprogramming following a primary immune stimulus that leads to increased (trained) or decreased (tolerant) responses to a secondary inflammatory stimulus (53). These concepts are important in the context of mild TBI as repeated head injuries lead to greater risk of poor outcome (32, 54). Although not defined as immune cells *per se*, astrocytes, oligodendrocytes, and endothelial cells all perform various functions that are critical to the immune response (55–58), and the important actions specific to these cells in mild TBI are reviewed elsewhere (59–62).

Neuroinflammatory cascades rely on the activation of the inflammasome, a protein complex, consisting of caspase-1, apoptosis-associated speck-like protein (ASC) and nod-like receptor protein (NLRP1 or NLRP3) (63, 64). Common microglial pathways activated upon the detection of a challenge involve NF-κB, which is a pro-inflammatory transcription factor that stimulates cytokine release in conjunction with the inflammasome (50). Metabolic changes within microglia also sustain or restrain inflammation (65). Rapid motility, reactive oxygen species (ROS) and cytokine production require quick energy utilization through glycolysis and fatty acid synthesis (65–67). In contrast, anti-inflammatory microglia require efficient energy production utilizing oxidative phosphorylation for transcription of ATP-dependent tissue repair genes, reduce ROS, perform amino acid and fatty acid oxidation to produce growth factors, including polyamines and prolines, and to support mitochondrial respiration (65–67).

In the event of CNS injury, microglia reduce their ramifications and extend cell processes to the site of injury, helping to maintain the integrity of critical CNS barrier structures such as glial limitans and vasculature (68–70). Moreover, they increase their migration to damaged brain sites and become phagocytic to clear cell debris (71–73). Microglia and other resident immune cells can be joined by their infiltrating counterparts from the circulation, such as neutrophils, monocytes, and lymphocytes, depending on the severity of injury (43, 71, 73–79). These peripheral cells are recruited through a multitude of mechanisms, including

endothelial and microglial signaling, and can enter the brain through the compromised blood brain barrier, circumventricular organs or other brain blood interfaces, such as the meninges (80, 81).

In the context of human mild TBI, it is unknown whether circulating immune cells are recruited to the brain in patients, and closed head animal models provide differing results depending on induction and severity of the injury [see below]. Recruitment of immune cells may vastly affect progression of pathology, as the actions of these cells can differ compared to their resident counterparts (43). Furthermore, evidence suggests that infiltrating immune cells influence resident microglia populations, which may have long lasting consequences for injury outcomes (74, 82–85).

Here, it is again important to distinguish between TBI with parenchymal hemorrhage (and associated animal models) and mild TBI, as hemorrhage is likely to create a type injury and immune response that is completely distinct to that of injuries with no bleeding (**Figure 1**). For example, extravasated red blood cells (RBCs) are a source of multiple immune response triggers and DAMPs (86). RBCs are phagocytosed by immune cells, which drives an inflammatory phenotype in those cells (87), and a portion of which stay within the tissue, die and degranulate, releasing endothelins and oxygen free radicals (88). Extravasated RBCs also releases toxic Hgb which is oxidized to haem and acts as a DAMP to exacerbate the inflammatory response (89). A further metabolite, iron is also implicated in brain injury after hemorrhage (88, 90, 91). Thus, several stages of RBC lysis contribute to a type of brain injury not seen in mild TBI without parenchymal bleeding. For information on the immune response to TBI including hemorrhage, we would like to point readers to the following excellent reviews on the topic (23–25).

In mild TBI, whether immune cells from the periphery are recruited or not (see below), microglia and the other resident immune cells are present and can respond rapidly to changes in the brain (44, 47–49). In the wider field of neuroimmunology, the interest in the microglia-mediated immune response during brain injury and disease has risen exponentially in the past decade, mainly due to genome wide association studies that implicate many microglial genes as risk factors for neurodegenerative disease (92, 93). It is here, in the brain's immune response, where mild TBI and the risk of cognitive decline and neurodegeneration may meet.

The Immune System's Role as a Driver of Neurodegeneration

To understand if the immune response to mild TBI increases the risk of neurodegenerative disease, it is important to understand the known role of the immune system in neurodegeneration. The CNS and innate immune system continuously modulate each other through a sophisticated bidirectional crosstalk (43, 94). Under pathological conditions, disrupted communication may result in an inflammatory response. When the inflammatory response of CNS resident immune cells remains unresolved, this may lead to initiation, propagation, and progression of tissue damage, ultimately resulting in neurodegeneration (50). The immune system may therefore be a driver of neurodegeneration, in general (95–98) and mild TBI's activation of the immune system may be a causative trigger, although this is yet to be formally demonstrated.

Many neurodegenerative disorders display concurrent and chronic alterations in immune function and signaling. However, there is now strong evidence that immune dysregulation can be a direct cause of neurodegenerative disease. Somatic mutation specifically in the erythro-myeloid progenitor lineage from which microglia derive can drive late-onset neurodegeneration in mice (56). More recently, biallelic mutations in *NRROS* (Negative Regulator Of Reactive Oxygen Species), which is necessary for TGFB-1 signaling in microglia, were found to cause an early onset lethal microgliopathy in humans (99, 100) and NRROS-deficient (Nrros-/-) mice show neurodegeneration (101), defects in motor functions and die before 6 months of age (102). Together, these data show that microglia-specific alterations can cause neurodegenerative disease, confirming that the well-documented immune response to neurodegeneration may not solely be secondary to injury.

Several genes involved in the immune system, and particularly microglia, have been identified as risk factors for the most common form of neurodegenerative disease, Alzheimer's (103–105). The APOE gene, encoding apolipoprotein (Apo)E (106) is mainly expressed in the brain by microglia and astrocytes and is component of Aβ plaques and promotes Aβ aggregation and deposition (107). The three major human isoforms, apoE2, apoE3, and apoE4, are encoded by different alleles and differ in their effects on AD risk and pathology, with one APOE-ε4 allele increasing AD risk 3-fold and two APOE-ε4 alleles increasing AD risk by 12-fold (108). In addition, APOE-ε4 is also implicated in Dementia with Lewy bodies and Frontotemporal dementia (97). Another molecule implicated in AD is the triggering receptor expressed on myeloid cells 2 (Trem2) and is highly expressed in microglia in the brain and important for microglial, phagocytosis, proliferation and environment sensing (109). Single-nucleotide polymorphism (SNP) mutations in Trem2 drastically increase the AD risk (110), estimated to be with a 3.0- to 4.5-fold (96). Moreover, homozygous Trem2 variants have been proposed to be causal for Frontotemporal Dementia (FTD) or linked to increased FTD risk (111). A recent genome-wide meta-analysis identified new loci and functional pathways influencing Alzheimer's disease risk that localized to immune-related tissues and cell types (microglia), highlighting the role of the immune system and its principle brain-resident cell in neurodegenerative disease (92). In sum, the immune system and its dysfunction are now strongly implicated in neurodegenerative disease, for which mild TBI (particularly repeated mild TBI) is proposed as a risk factor. We will now summarize how mild TBI may activate the brain's immune system, potentially linking it to long-term neurodegeneration.

THE IMMUNE RESPONSE TO MILD TBI

To reiterate the focus of this article, we will review the known immune response to injuries that are defined as mild TBI

or concussion and their relevant animal models. This would therefore exclude brain injuries with intracerebral, subdural, epidural hematoma, cerebral or hemorrhagic contusion, penetrating TBI (dura penetrated), subarachnoid hemorrhage or brainstem injury (2) (**Figure 1**). This definition describes a population of patients that have potential pathology too subtle for standard imaging, but represents up to >80% of those that suffer a TBI (3). Animal models representing this "mild" situation are less common as researchers investigating brain injury are in search robust, reliable, modifiable readouts to demonstrate mechanisms and new treatment options; such animal models often employ craniotomy and/or hemorrhage and contusion and would be considered moderate to severe TBI in the clinic. If the immune response to mild TBI is a factor in pathology, it is important to understand how this may be initiated in a brain without bleeding or overt lesion.

Triggers of the Immune Response in Mild TBI

The immune system is ubiquitous in the CNS and is equipped to respond to brain tissue injury after mild TBI. The injury produced after a closed-head impact is the result of physical movements of the brain within the skull, including acceleration and differential inertial loading (10, 11). Physical forces are thought to lead to axonal injury due to excessive regional stretching of axons (112) (**Figure 2A**). Using head kinematics from athletic events, modeling showed that the brain can be described as a hyperviscoelastic medium and deformation is most sensitive to specific frequency oscillations, particularly in deep brain regions, and is aligned with areas of pathology after mild TBI (10).

Stretching of axons is the leading hypothesis as to why white matter appears most sensitive to mild brain injury. However, from the view of the immune system, microglial cells are also sensitive to mechanical signals (113, 114). Viscoelastic testing of individual CNS cells showed that other glial cells, such as astrocytes and Müller glia are twice as soft as neurons and act as compliant structures surrounding the neuronal cells, and are described as "cushioning material" (115). This opens the possibility that glia, in general, are differentially susceptible to forces produced in the brain during mild TBI. Mechanical changes in glial cells may physically protect neurons from initial mechanical damage, but then subsequently become activated and produce factors that drive neuronal pathology (**Figure 2A**). Alternatively, glia may be bystanders, solely responding to neuronal injury when the force is big enough to affect neurons directly. In either scenario, an aberrant microglial response due to either genetic factors, such as APOE genotype or TREM2 mutations (see above), or environmental influences, such as inflammation due to infection, poor diet or obesity may contribute to concussive injury as a risk factor for neurodegenerative disease (26).

Physical forces may also result in damage to the blood-brain barrier (BBB), a specialized endothelial barrier that tightly regulates molecular and cellular movement into the brain (116). BBB breakdown is long associated with moderate to severe brain injury (**Figure 2B**) but has recently been shown to be present in clinically relevant models of mild TBI. In awake mice, 24 h after a closed-head impact injury, serum albumin extravasation and evidence of myeloid inflammatory cell infiltration due to BBB breakdown was localized to the lateral surface of the ipsilateral perirhinal cortex adjacent to the impact contact zone (117). In a swine model of head rotational acceleration and in the absence of hemorrhage or other focal pathology, disruption of the BBB was found 6–72 h after injury, by extravasation of serum proteins, fibrinogen and immunoglobulin-G (12), confirming earlier mouse studies (118). BBB disruption was consistent with the biomechanical insult as extravasated serum proteins were observed at interfaces between regions of tissue with differing material properties, including gray–white matter boundaries and periventricular and subpial regions which overlapped with regions of axonal pathology in the white matter (12). This highly relevant model of mild TBI provides insights to subtle, yet significant pathology that is likely to trigger immune responses, yet be undetectable in humans through standard clinical imaging techniques.

Fibrinogen is a central blood coagulation protein that is deposited in the CNS after BBB disruption (62, 119) and is found in brain tissue in models of mild TBI described above (12). Fibrinogen induces encephalitogenic adaptive immune responses and peripheral macrophage recruitment into the CNS leading to demyelination in models of multiple sclerosis (120). Interestingly, fibrinogen deposition in the CNS affects many processes across diseases, including suppression of remyelination through OPC function (121) and induction of microglia-mediated spine elimination, leading to cognitive deficits in a model of AD (122). Both white matter damage and cognitive deficits are described in mild TBI. Currently, a promising advance for treating pathologies in involving fibrinogen deposition, is the generation of a monoclonal antibody 5B8, that selectively inhibits fibrin-induced inflammation and oxidative stress without interfering with clotting and shows efficacy in animal models of MS and AD (123). Whether such strategies will be employed to improve pathology and cognitive and emotional deficits after mild TBI remains to be seen.

More data are required to understand the initial triggers of the immune response in mild TBI, and a combination of neuronal damage through mechanical stretching, direct mechanical damage of glia and endothelial cells and BBB-breakdown are all likely to play a role. Irrespective of the initial driver of brain tissue damage, patient studies indicate there is an active immune response in those individuals exposed to mild TBI injury.

Clinical Evidence of the Immune Response to Mild TBI–Serum Biomarkers

An important area of brain injury and disease research is the search for blood biomarkers that reflect brain processes, to predict disease course and used as readouts to assess utility of interventions to improve outcome for patients. Recently, a range of proteins, including neurofilament light (NfL) polypeptide released from damaged axons, have been proposed as viable biomarkers of mild TBI (60, 124, 125). The brain's immune

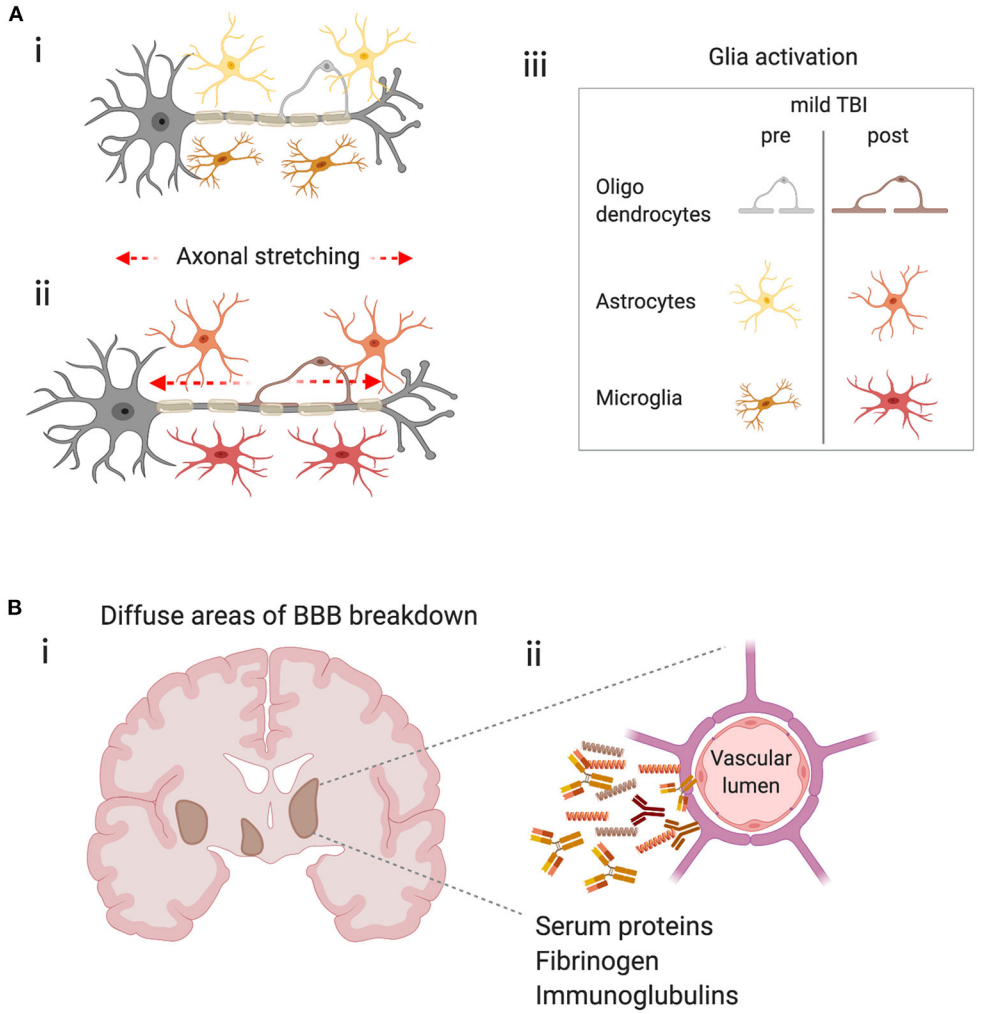

FIGURE 2 | Potential triggers of the immune response in mild TBI. **(A)** Physical forces lead to stretching of axons, axonal injury and glial activation. (i) Schematic representing a neuron with its myelinated axon surrounded by glia in the healthy brain. (ii) Represents the stretching of axons due to physical forces during mild TBI and the subsequent glial activation (iii) Key–indicates glial subtypes hypothesized to be activated after axonal stretching. **(B)** Diffuse blood-brain barrier (BBB) in the brain after concussion leads to extravasation of harmful molecules to the parenchyma. (i) Schematic shows a coronal section of a human brain with representations of diffuse BBB breakdown after mild TBI (brown areas). (ii) Enlarged image shows compromised cerebral blood vessel leaking molecules that may trigger inflammation and glial activation.

response to mild TBI is also evident in the blood, as elevated c-reactive protein (CRP) levels at admission are independently associated with the increased risk of persistent psychological problems and cognitive impairment (29). Plasma interleukin (IL)-2 and IL-6 levels are also significantly higher for mild TBI patients compared with orthopedic injury controls, indicating a brain-specific injury initiated an immune response that is present in the periphery (126). Elevated IL-2, 24 h after injury, is associated more severe early post-concussive symptoms, while elevated plasma IL-10 level at 6 months is associated with more severe posttraumatic stress disorder (PTSD) and mood scores (126). Overall plasma levels of IL-1ß, IL-4, IL-6, and IFN-γ are reduced at 6 months compared to acute levels, indicating a subsiding of inflammation caused by the initial injury. Interestingly, plasma levels of the anti-inflammatory cytokine IL-10 have also been shown to be predictive of a mild TBI vs. a moderate-to-severe TBI (as defined by hemorrhage on CT) (127), again highlighting the different immune responses between injury types. Further to immune mediators detected in the plasma, complement pathway proteins (key mediators of the immune response) are elevated in astrocyte-derived exosomes in the plasma within seven days of a mild TBI (128). In the non-acute setting, a study of veterans with a remote history of mild TBIs found an association between concentrations of TNF-α and post-concussive syndrome (PCS) and PTSD symptoms (125). The total number of mild TBIs correlated with exosomal and plasma NfL levels and plasma IL-6 (125). These results indicate a persistent elevated

neuronal and neuroinflammatory response many years after mild TBI.

Serum biomarkers of brain injury and the immune response are now being collected in a range of settings, from single injuries to chronic sport related mild TBI in male and female participants (129). Although, the peripheral immune response to more severe TBI is well documented (24, 64, 130), the data specifically in mild TBI highlights that circulating immune mediators are also present and may be involved in symptom progression and resolution.

Clinical Evidence of the Immune Response to Mild TBI–Neuroimaging

The immune response can also be measured indirectly in the brain by clinical neuroimaging. The meninges, a protective layer of membranes surrounding the brain, have gained much attention in the field of neuroimmunology as they are the home of the brain's lymphatic system (131, 132) and a variety of immune cell populations (44, 47, 49) which, in animal models, can regulate brain function in health (133), disease (134, 135), infection (136), and recovery from TBI (137). In patients with mild TBI, enhancement of the meninges on post-contrast images obtained by fluid attenuated inversion recovery (FLAIR) magnetic resonance imaging (MRI), show abnormalities that may reflect inflammation in the immune cell rich meningeal membranes (138, 139). Meningeal immune cell-mediated inflammation can influence neuronal populations in the brain parenchyma in mice, resulting behavioral changes that are also associated with symptoms of mild TBI, such as anxiety (133, 137). As yet, it is unknown whether meningeal inflammation in human mild TBI plays a role in emotional symptoms seen after injury.

In the context of athletes with a history of mild TBI, initial neuroimaging studies of former retired NFL players reported cognitive deficits that were correlated with white matter abnormalities and changes in regional cerebral blood flow (140) and that mild TBI is a risk factor for development of mild cognitive impairment (140). Following these findings, work began to show that neuroinflammation in the brain tissue proper was also a key component in those at risk from concussive-symptoms.

It is well-known that brain injury and disease cause a change in the functional state of microglia, the major cell type in the brain responsible for neuroinflammation (141). Neuroinflammation is associated with the *de novo* expression of the mitochondrial 18 kDa translocator protein (TSPO), a binding site for which many selective high-affinity compounds for PET imaging have been developed (142). During brain injury or disease, TSPO is mainly expressed in microglia but can also be detected in astrocytes (51, 143, 144). Increased regional TSPO expression in the brain typically covaries with disease state and activity and is proposed to be a non-diagnostic biomarker and secondary to disease etiology. As result, many clinical studies use *in vivo* measurements of TSPO expression as a biomarker of disease progression or therapeutic efficacy (142).

In former NFL players, early studies showed significant increase in binding of the radio-ligand [^{11}C]DPA-713 to TSPO in several brain regions, such as the supramarginal gyrus and right amygdala, compared to age-matched, healthy controls, indicating neuroinflammation in those areas (145). The same former players had varied performance on a test of verbal learning and memory (145). Interestingly, studies performed in much younger, active and recently retired NFL players with a self-reported history of mild TBI also revealed increased [^{11}C]DPA-713 binding to TSPO in eight of the 12 brain regions examined, but did not differ from control participants in regional brain volumes or in neuropsychological performance (146). This opened the possibility of using TSPO binding a biomarker for brain changes in the younger concussed brain, prior to cognitive decline (146). Postmortem studies of former NFL player confirmed that repeated head injury is associated with chronic activation of microglia (147). In addition, the duration of repeated head injury exposure, as defined by the years of football played, predicted greater density of CD68 positive inflammatory microglia in NFL players with and without CTE pathology and that increased neuroinflammation was related to the risk of a subject being diagnosed with dementia, independent of age (147). Together, these studies show that the immune response is detectable in patients before cognitive changes, and persists throughout the lifetime of those exposed to concussive injury.

Other strategies to assess immune cell activation in response to concussion injury include the quantification of protein markers in the CSF, such as soluble TREM2 (sTREM2). TREM2 is a transmembrane receptor of the immunoglobulin superfamily highly expressed in microglia which multiple ligands (148). TREM2 surface expression rapidly declines on activation of microglia which is partly due to sequential cleavage of membrane-bound TREM2 by proteases that release soluble TREM2 (sTREM2) (149). As result, sTREM2 has been hypothesized to be predictive of microglial activation if detected in the CSF. In former NFL players, sTREM2 levels were higher in their CSF compared to controls and were associated with higher t-tau concentrations, which are thought to be a major factor in neurodegeneration (150). However, in patients with TREM2 mutations with AD and FTD, sTREM is reduced in the CSF (151). Other studies have shown increases of CSF TREM2 in AD dementia (152, 153), or increase in MCI-AD but not AD dementia (154). Therefore, the use of sTREM as a direct biomarker for microglia activation is yet unproven, and the biological role sTREM2 in neurodegeneration is unknown.

Patient studies investigating the microglial response to mild TBI have largely focused on individuals with a known history of head injury, such as professional athletes. Recently, TSPO expression was assessed after a single-event mild TBI in patients without signs of structural damage 1–2 weeks and at 3–4 months after injury (155). Importantly, patients were not included if they showed any intracranial lesion on the initial computed tomography scan or had a history of head trauma with loss of consciousness. Using the single photon emission computed tomography tracer 123I-CLINDE, persistent TSPO upregulation was found at 3–4 months post-injury, even in patients with good clinical recovery (155). This is consistent with the data

from active or recently retired NFL players that had increased TSPO binding, but no change in neuropsychological testing (146). Clearly, it is unknown whether a single head injury and subsequent TSPO increases is comparable to a history of head injury and persistent TSPO increases, and whether they have the same long-term consequences, but it is evidence that a sub-chronic immune response occurs in even mild injury with resolved short-term clinical symptoms.

The Immune Response in Animal Models of Mild TBI

Models of traumatic brain injury have provided insight into multiple mechanisms of tissue damage. Even for "mild" TBI, animal models vary widely, from impacts direct to the brain's surface in fixed-head, anesthetized animals to injury with no surgery or anesthesia with forces that allow head rotation (59, 156). Each model (even similar in description), in different laboratory's hands can lead to different etiologies and severities, which should be considered when assessing the literature. Often the least "clinically relevant" model can lead to the most useful mechanistic insight, and those most likely to best recapitulate human concussion should inherently be the most heterogeneous in their outcome. Both extremes are now being used to explore the role of the immune system in mild brain injury. We will not attempt to review the different models of injury here, but we point the reader to excellent reviews in the field (59, 156). As mentioned previously, we will focus our attention on closed head injury models that most closely resemble clinical mild TBI, at least in as much as do not use any type of craniotomy or produce frank tissue damage or hemorrhage. We will assess the only those models that are "closed skull" with no observable bleeding and investigate the immune reaction to injury. These models are relatively few compared to the wider TBI literature but may offer key insights to pathology.

In a pig model of closed-head rotational acceleration, biomechanical loading parameters can be replicated that are thought to underlie neuropathology of mild TBI in humans (157). Pigs have a large gyrencephalic brain and a gray to white matter ratio similar to humans, which is important as diffuse axonal injury in white matter is believed to be the principal pathology of closed-head diffuse brain injury (42, 158). Using these models, changes in microglia morphology around compromised neurons was described as early as 15 min after injury, potentially allowing microglia to influence the evolution of subsequent neuronal damage (159). Evidence from many other models of CNS injury suggest that initial actions of microglia are protective (69, 160–162), though this is unknown in the context of mild TBI. In the same pig model, investigation of later time points after injury, found subtle neuronal changes in the hippocampus (163, 164) and microglia had increased signs of activation up to 1 year after injury (164). Just as acute microglial actions at sites of CNS injury are proposed to be beneficial, long-term microglial-mediated inflammation is thought to be detrimental and may be involved in the long-term complications associated with mild TBI (165–173). Longer-term activation of microglia was also seen in non-surgical, diffuse closed-head injury in mice, in a model characterized by an impact as well as linear and rotational acceleration (174). Thirty days after injury, multifocal, bilateral axonal damage with neuronal death in the hippocampus was detected, microgliosis was prominent and neurobehavioral deficits observed in spatial learning/memory and socialization (174). Indeed, a range of studies in appropriate rodent models show microglial activation across multiple time points, particularly in white matter tracts (165–172) and may be a result of rotational stress or reduced cerebral blood flow (175, 176).

To investigate the causality of inflammation and the immune response on mild TBI deficits, multiple studies have manipulated these pathways and investigated behavioral outcome measures. For example; selective, small molecule inhibition of acute pro-inflammatory cytokines and chemokines, nanopeptides targeting apoE mediated the neuroinflammation and minocycline administration all reduce microglial activation and improve neurological outcome after mild TBI, respectively (177–179). Hippocampal microglia activation is attenuated by inhaled nitrous oxide and correlates with improved performance on memory tasks (180) and statins reduce pro-inflammatory cytokine gene expression in the brain, reduce microglial activation and improve functional neurological deficits after mild TBI (181). Nilvadipine, a tyrosine kinase inhibitor, suppresses inflammation and restores spatial memory deficits (182) and administration of Carnosic acid, and inhibitor of NF-kB, significantly improves motor and cognitive function and reduces microglia activation in white matter tracks in a mouse models of repetitive mild TBI (183). The neuronal expression of pro-inflammatory mediator complement receptor C5a is upregulated after mild TBI and is dependent on TNF (184). Mice lacking a functional alternative pathway of complement activation have reduced neuronal cell death after mild TBI (185) and removal of the protective neuronal-derived complement protein CD59, worsens neurological outcome seven days after mild TBI in mice (186).

These studies show that, like in humans, inflammation and microglial activation are prolonged after injury. Animal models show that the neuroimmune response correlates with cognitive deficits and may be modified to improve outcome, though more evidence of causal contribution of the immune system to behavioral deficits is needed.

Models of mild TBI are continually being refined. Recently, it was shown that a commonly used inhaled anesthetic, isoflurane, inhibits microglial ramification and surveillance *in vivo* (187), therefore potentially blocking the immediate immune response of the brain to injury when animals and anesthetized. The issue of anesthesia is prominent in almost all models of brain injury, but some have begun to produce concussive injuries in awake, restrained animals. In awake rats, using an injury paradigm with a bespoke helmet, injury produced memory deficits, and microglia activation after impact verses sham (188). Although, these studies remove the issue of anesthetic effects on the immune system, the stress induced by restraint of animals must be considered. Restrain habituation for the above study was performed for 3 days before injury, and stress induced inflammation may still be a contributing factor.

The onset and duration of inflammation driven by immune mechanisms after concussion is likely to play a key role in pathology. Activation of Toll-like receptor 4 (TLR4), which

plays a fundamental role in pathogen recognition and activation of innate immunity, following repeated mild TBI is either beneficial or detrimental depending on the timing of activation. Administration of low dose LPS 1 day after injury was associated with a reduction in neuronal injury, a restoration of levels of myelin basic protein (MBP) and PSD-95 and no behavioral changes in locomotion, anxiety, depressive-like behavior or cognition at 3 months post-injury (189). Conversely, when LPS was given at 5 days after injury, it was associated with an acute increase in pro-inflammatory cytokine production, an exacerbation of neuronal damage and increased levels of aggregated and phosphorylated tau which led to a slight exacerbation of cognitive deficits and depressive-like behavior at 3 months (189). Due to the interest in the immune response after mild TBI, a natural avenue for therapeutics are anti-inflammatory treatments. However, modulating the immune response may require a strict, and yet unknown, time course. The strength of immune modulation should also be considered. In a model of AD, it was found that targeting the TLR4 receptor before onset of pathology by inducing either immune tolerance (4xLPS injections) or immune training/priming (1xLPS injection), either alleviates later B-amyloidosis with the former, or makes it worse with the latter (190). Such mechanisms must be considered when approaching inflammation after mild TBI, especially considering mild head injury's convergence with neurodegenerative disease (89).

In a model of lateral closed-head impact injury that uses momentum transfer to induce traumatic head acceleration in unanaesthetised mice, an abrupt onset and rapid resolution of a concussion-like syndrome is characterized by altered arousal, locomotor impairments and neurobehavioral deficits (117). The majority of brains in injured mice (∼90%) had no evidence of frank hemorrhage or contusion but BBB breakdown was observed. An increase in the number of microglia and in infiltration of monocytes was observed 3 days after injury, though this was localized to the impact region (117). Uniquely, the study presented post-mortem brains from four teenage athletes in the acute-subacute period after mild closed-head impact injury and found, among other pathology, perivascular neuroinflammation in the form of haemosiderin-laden macrophages surrounding a small blood vessel (117). These data indicate that microglia may not be the only immune cell contributing to mild TBI pathology, as monocyte-derived macrophages from the circulation may enter brain tissue after mild injury, though more data is required to confirm this as an immune response to mild TBI pathology, in general.

Repeated mild TBIs cause more significant neurological damage than a single injury, including longer recovery time and a higher likelihood of subsequent brain injury (32, 54). It is hypothesized that priming of the immune response may play a role. Repeated mild brain injury produces greater microglial activation, anxiety-like behavior and impaired spatial memory compared to a single injury, in mice (191). In rats tested with projectiles and helmets, repeated injury produced a more significant and long lasting inflammatory response associated with microglial activation than a single injury (192). Delivering multiple mild impacts over a shorter inter-injury interval leads to a more significant acute microglial activation and prolonged astrogliosis in select regions of the brain, compared to the same number of administered over a longer time-period (193). The corpus callosum, hippocampus and lateral septum appear particularly vulnerable to injury (191, 193) and these areas may contribute to clinical symptomology, including anxiety and memory problems (191, 193). Frequent mild head injury also promotes trigeminal sensitivity with associated microglial proliferation and increased neuropeptide levels in the trigeminal pain system (194), which is associated with headaches and migraine that accompany post-concussion syndrome. In a mouse model of highly repetitive mild TBI, 30 injuries cause white matter pathology, and microglial proliferation and activation. This pathology is present 60 days after final injury, and is still apparent at 1 year (195).

The Immune Response in Animal Models of Mild TBI and Alzheimer's Disease

As discussed above, epidemiological studies associate increased risk of AD-related clinical symptoms with a history of mild TBI (2, 26, 33, 35). To investigate the link between mild TBI and AD, studies have performed brain injury in genetic mouse models of AD and assessed the neuroinflammatory response driving pathology and clinical symptoms (196–200). In APP/PS1 mice that contain human transgenes for both mutated APP and PSEN1 (201), neuroinflammatory gene expression is increased seven days after injury and microglia activation is greater at 2 months in APP/PS1 compared to WT mild TBI controls (197). Importantly, a small molecule inhibitor, previously described to selectively limit pro-inflammatory cytokine production after mild TBI (202), improved cognitive behavior outcomes in APP/PS1 mice after injury (197). Thus, providing a link between neuroinflammatory responses and altered risk for AD-associated pathology changes after mild TBI (197). Other studies in APP/PS1 mice and mild TBI models have investigated acute neuroinflammatory outcomes in young and old mice, however, the interplay between the immune response and AD-like progression was more complicated as neuroinflammation is increased in aged WT mice but reduced in aged APP/PS1 mice (199, 200). In a repeated mild TBI model (12 injuries in 1 month) and aged APP/PS1 mice, there was no increase in various makers of microglia activation 1 month after final brain injury compared to sham control (203). However, there was an increase in brain insoluble to soluble Aß42 ratio in injured APP/PS1 mice compared with sham and a parallel reduction in phagocytic receptor, TREM2, suggesting pathology mediating microglia changes induced by injury (203). In a transgenic mouse model for human tau (hTau), acute microglia activation after mild TBI is increased in both young and aged animals, however, this response is not as robust in aged animals compared to young, suggesting a diminished acute microglial response to mild TBI in animals with established AD pathology (204) and highlights the complicated nature of priming and tolerance in chronic neurodegenerative disease (53).

To model subthreshold injury be expected in competitive contact sports, a 7 days 42-impact paradigm with helmets in

mice was used to simulate frequent head injury (196). This paradigm resulted in chronic gliosis and T-cell infiltration of the superior colliculus and optic tract, with concomitant demyelination of the optic nerve and associated white matter tracts 1 month after injury (196). When injuries were performed in Tau58.4 mice, there was progressive neuroinflammation and neurodegeneration in multiple brain regions compared to WT mice. To investigate the role of T-cells in these specific areas vulnerable to demyelination, T-cell-deficient Rag2/ Il2rg (R2G2) mice were subjected to the same injury paradigm. R2G2 mice had altered myeloid cell gene expression and fewer demyelinated lesions compared to T cell-competent mice. This study suggests that vulnerable regions known to be affected in CTE, such as white matter, may be protected by manipulating the immune system (196).

In summary, animal models of mild TBI are varied, but extremely powerful tools investigate mechanisms of injury. There is now a wealth of evidence linking the immune response to mild TBI and the behavioral and pathological outcomes. This is fueling many investigations into new therapeutics targeting these mechanisms.

CONCLUSION

Mild TBI, often referred to as concussion, is the most common form of traumatic brain injury and can result in insidious effects including emotional and cognitive dysfunction. In addition, a history of mild TBI is proposed as a risk factor for longer term neurodegenerative disease. Despite this, the underlying pathology is still unclear. Immune activation, and particularly changes to microglia are associated with human mild TBI and a variety of animal models of mild brain injury. In many cases these are descriptive, although evidence suggests immune activation correlates with cognitive and behavioral symptoms. Animal studies are beginning to demonstrate a causative role of the immune system in acute brain dysfunction following mild TBI. The fact that the immune system is readily reactive, and can remain active over a long period of time after injury, leaves open the possibility that an aberrant immune response, driven by factors that have shown to be important for neurodegenerative disease, may contribute to the long-term consequences of mild brain injury.

AUTHOR CONTRIBUTIONS

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T Cells Actively Infiltrate the White Matter of the Aging Monkey Brain in Relation to Increased Microglial Reactivity and Cognitive Decline

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Normal aging is characterized by declines in processing speed, learning, memory, and executive function even in the absence of neurodegenerative diseases such as Alzheimer's Disease (AD). In normal aging monkeys and humans, neuronal loss does not account for cognitive impairment. Instead, loss of white matter volume and an accumulation of myelin sheath pathology begins in middle age and is associated with cognitive decline. It is unknown what causes this myelin pathology, but it likely involves increased neuroinflammation in white matter and failures in oligodendrocyte function (maturation and repair). In frontal white matter tracts vulnerable to myelin damage, microglia become chronically reactive and secrete harmful pro-inflammatory cytokines. Despite being in a phagocytic state, these microglia are ineffective at phagocytosing accruing myelin debris, which directly inhibits myelin sheath repair. Here, we asked whether reported age-related increases in pro-inflammatory markers were accompanied by an adaptive immune response involving T cells. We quantified T cells with immunohistochemistry in the brains of 34 cognitively characterized monkeys and found an age-related increase in perivascular T cells that surround CNS vasculature. We found a surprising age-related increase in T cells that infiltrate the white matter parenchyma. In the cingulum bundle the percentage of these parenchymal T cells increased with age relative to those in the perivascular space. In contrast, infiltrating T cells were rarely found in surrounding gray matter regions. We assessed whether T cell infiltration correlated with fibrinogen extravasation from the vasculature as a measure of BBB leakiness and found no correlation, suggesting that T cell infiltration is not a result of passive extravasation. Importantly, the density of T cells in the cingulum bundle correlated with microglial reactivity and with cognitive impairment. This is the first demonstration that T cell infiltration of white matter is associated with cognitive decline in the normal aging monkey.

Keywords: myelin, T cells, aging, cognitive decline, microglia, blood brain barrier, white matter

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INTRODUCTION

Even in the absence of pathologic neurodegeneration, impairments in learning, memory, executive function, and processing speed begin as early as the third decade (1) making cognitive decline a hallmark of both healthy and neurodegenerative brain aging. In addition, there is much inter-individual variability in the severity and age of onset of these cognitive changes, with some individuals aging very successfully but about 30% of individuals suffering severe cognitive impairment without meeting the criteria for Alzheimer's disease or clinical dementia (2). The cognitive decline of normal aging cannot be attributed to neuronal loss, as neuronal density remains stable across the lifespan (3). Instead, studies using MRI and tissue analyses have found that white matter volume declines with age and correlates with cognitive impairment in healthy aged adults (4–7). Notably, damage to white matter tracts does not occur equally throughout the brain as diffusion tensor imaging has shown that, in aging humans, disruption occurs first and to a greater degree in tracts of the frontal white matter as well as the cingulate bundle compared to tracts of the occipital and temporal lobes (8–12).

The rhesus monkey is a widely used model for studying normal aging because they exhibit cognitive decline in the same domains as humans and have similar proportions of successful and unsuccessful agers (13). Extensive cognitive testing in our monkey model of normal aging has demonstrated age-related impairments in learning, memory, and executive function (14). Moreover, rhesus monkeys do not develop Alzheimer's or other neurodegenerative diseases and both gray matter volume and neuron numbers are stable across the lifespan (14–17). Thus, the rhesus monkey can serve as a model for the age-related cognitive impairment of normal aging without the confounds of occult neuropathology that challenge human studies. Importantly, rhesus monkeys also have a ratio of white to gray matter similar to humans and exhibit an age-related decrease in white matter volume and increase in myelin damage which correlate with cognitive decline (17–19). Diffusion imaging in rhesus macaques confirms the same differential vulnerability of white matter tracts observed in humans, with frontal tracts exhibiting disruption while other white matter tracts such as the internal capsule remain stable with age; and these tract disturbances correlate with cognitive impairment (20). Ultrastructural examination of our monkey brains has demonstrated that white matter pathology begins with splitting of the sheath and formation of balloons filled with degenerating cytoplasm or fluid (21, 22). Such defects are present in <1% of the sheaths in young monkeys but increase 7-fold to 7–8% in the oldest monkeys and correlate with cognitive impairment (19). These ultrastructural studies have confirmed the vulnerability of frontal white matter tracts to myelin sheath damage (19, 21, 23). Additionally, this pathology is accompanied by axon degeneration, albeit at a lower rate (0.1–0.8%) (19) so that it is likely that the observed myelin pathology impairs axon conduction producing a disconnection that likely contributes to cognitive impairment (18, 22, 24).

While the exact causes of myelin pathology in aging are unknown, several different processes may play important roles. Myelin maintenance or homeostasis relies on a complex interplay of oligodendrocyte maturation, myelin plasticity, removal and clearance of myelin debris and remyelination—processes that all are negatively affected by aging (25–29). A failure of oligodendroglia precursor cells (OPCs) to mature into myelinating oligodendrocytes results in decreased remyelination in an animal model of demyelination (30) and aged rodents exhibit a decreased ability to replenish their OPC population, which may partially underlie age-related failure of myelin repair (29). Aging humans exhibit a 34% decrease in the number of oligodendroglia with age, while neurons, microglia, and astrocytes remain relatively stable across the lifespan (25). Microglia play a critical role in promoting oligodendrocyte precursor cell differentiation into mature myelinating oligodendrocytes (31) as this maturation is inhibited by the presence of myelin debris in the local neuro-environment (32)—debris that is normally cleared by microglia. Thus, microglia play a multifaceted role in promoting remyelination by oligodendroglia (33). With age, microglia become laden with lipofuscin, a remnant of phagocytosed material that accumulates and can interfere with the cell's ability to ingest more debris (34). With age, microglia are rendered less effective in clearing myelin and become chronically reactive as indicated by the increased density of amoeboid and hypertrophic microglia in the aged brain, which correlates with the severity of cognitive decline in aged monkeys (35). Along with failing to clear myelin debris, aged microglia are also characterized by an increase in the secretion of pro-inflammatory cytokines which are known to negatively impact cognition and increase production of reactive oxygen species (ROS) that can directly act to breakdown the lipids of the myelin sheath (36, 37). Together these data suggest that microglia, the main immune system cell of the brain, may contribute to age-related myelin pathology and white matter loss.

In addition to their likely role in exacerbating myelin damage, senescent microglia contribute to the more general phenomenon known as "inflammaging;" a term that has been used to describe the chronic increase in pro-inflammatory cytokines and chemokines in the aging CNS (38, 39). Systemic inflammation increases with age and is believed to be driven by dysregulation of the innate immune system, specifically by means of monocyte over-activation and secretion of pro-inflammatory molecules (40). Age-related neuroinflammation has been documented across species from rodents to humans and has been shown to be associated with normal cognitive impairment as well as age-related neurodegenerative diseases (41–43). In aging rodents, increased secretion of pro-inflammatory cytokines is accompanied by a decrease in cognitive performance, while those animals with more anti-inflammatory cytokines remain more cognitively spared (43). It has been demonstrated that pro-inflammatory cytokines underlie decreased secretion of neurotrophins and inhibit neurogenesis (44, 45). In our primate model of normal aging and white matter degeneration, we have shown that microglia exist in a chronic activation state and exhibit signs of increased phagocytic activity, states that correlate with severity of cognitive impairment (35). While changes to microglia, cells of the innate immune system, in brain aging

have been characterized, less is known about how cells of the adaptive immune system, such as T cells, might be involved with CNS aging.

Conventional wisdom regarding immune privilege of the brain held that cross-talk between the CNS and the peripheral immune system was largely restricted by the blood brain barrier (BBB) (unless it was damaged by disease or trauma), preventing immune cells of the peripheral blood from entering the CNS. Reexamination of these issues has shown that CNS-antigen specific T lymphocytes reside at CNS border zones such as in the meninges and choroid plexus and exert a variety of effects on the brain including playing a critical role in normal cognitive function (46, 47). Thus, until recently, it has been thought that T cells of the peripheral immune system only cross the healthy BBB to survey the brain environment for infection, after which they exit back into the peripheral circulation *via* lymphatic drainage of the brain (48). With age, T cells at the choroid plexus undergo a shift from a more homeostatic profile to a more detrimental proinflammatory profile, which is negatively associated with cognition (49, 50). Moreover, a recent study also demonstrated T cell residence in the aging brain parenchyma where they have been shown to play a negative role in cognition by inhibiting hippocampal neurogenesis (51).

To determine if T cells are involved in white matter aging, we examined a cohort of 34 rhesus monkeys of different ages and both sexes that were cognitively characterized demonstrating varying degrees of age-related cognitive impairment (**Figure 1B**). Previous studies have shown that myelin sheath damage, particularly in the frontal white matter was the best predictor of age-related cognitive impairment (19, 21, 23, 52). Microglial activation and phagocytic dysfunction specifically in the aging white matter correlate with cognitive impairment severity and are hypothesized to play a central role in the age-related impairments in myelin sheath homeostasis (34, 35, 53–55). In the present study, we specifically asked whether myelin damage and chronic microglial activation in the frontal white matter of our aging monkeys are accompanied by a peripheral immune response of T cells. We demonstrate that not only do T cells surrounding blood vessels increase with aging, but T cells also infiltrate the white matter parenchyma where they correlate with the degree of microglial reactivity and cognitive impairment. Here, we present the foundation for examining T cells as a novel player in normal age-related cognitive decline.

METHODS

Subjects

Male and female rhesus macaques aged 5–30 years old—equivalent to human ages 15–90 years old (56)—were carefully selected to exclude subjects with comorbid disease or experimental manipulations that would confound studies of the aging brain and behavior (**Figure 1A**). While in the study, subjects were maintained in the Animal Science Center on Boston University Medical Campus (BUMC), which is fully accredited by AAALAC and managed by a licensed veterinarian. All procedures conformed to the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Use and Care Committee (IACUC) of BUMC.

Behavioral Testing and Cognitive Impairment Index

Monkeys received a behavioral test battery to assess learning, memory and executive functions. This battery consists of delayed non-match to sample (DNMS), delayed recognition span (DRST), both object and spatial modalities, delayed response, and conceptual set-shifting tasks. These tasks are briefly detailed below and more detail can be found in (14, 57, 58), as well as a summary of how our cognitive impairment index is calculated from a subset of these tasks (13). Testing was conducted 5 days a week and used food rewards. Water is available *ad libitum* and a daily ration of chow, fruits, and vegetables is given each day after testing is complete.

Delayed Non-match to Sample (DNMS) Task

The DNMS is a benchmark task of learning and recognition memory which measures the monkey's ability to distinguish between a recently presented familiar object and a novel object following a delay period of 10 s. Once this is learned additional tests of recognition memory after done after delays of 2 min and then 10 min. Output of the acquisition phase, 2-min delay phase, and 10-min delay phase are used as measurements for this task of learning and recognition memory.

Delayed Recognition Span Task (DRST) Object and Spatial

This tests monkeys' working memory capacity by requiring that the monkey to identify a new stimulus among an increasing array of serially presented, familiar stimuli first using spatial then non-spatial (objects) stimuli. Output is the span of correct responses across trials as a measurement of working memory.

The Delayed Response (DR) Test

This tests monkeys' spatial working memory by assessing the monkey's ability to correctly identify, following various delays, the location of a food reward they previously saw hidden.

Conceptual Set Shifting Task

This tests the monkeys' executive functioning by having the monkey learn rules not explicitly learned. Much like the Wisconsin Card Sorting Task, once the task is learned, the "rule" is switched and the monkey must shift to learn the new rule. Output is based on the errors the monkey makes and specifically the perseverative errors made after set shifting as a measure of executive function.

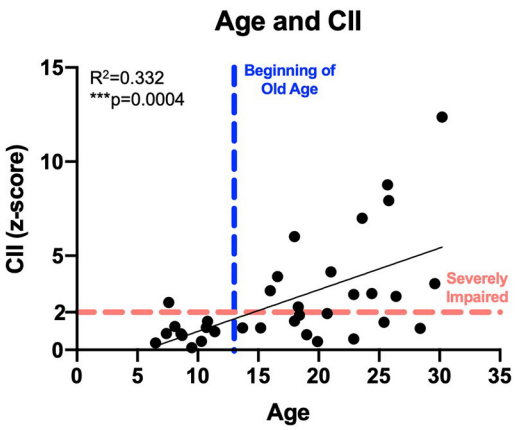
Calculation of Cognitive Impairment Index (CII)

A principal components analysis revealed that scores on DNMS-acquisition, DNMS-2-min delays, and DRST-spatial were the best predictors of age-related cognitive impairment. From this subset of scores, a weighted average is computed and converted into a *z*-score that is normalized to the mean performance of a cohort

A

AM	AGE	SEX	CII
AM188	6.5	F	0.364
AM222	7.4	M	0.871
AM292	7.6	M	2.515
AM280	8.1	M	1.244
AM299	8.6	F	0.85
AM296	8.7	M	0.774
AM255	9.5	F	0.123
AM202	10.3	F	0.451
AM214	10.7	F	1.199
AM295	10.8	M	1.523
AM254	11.4	F	0.972
AM291	13.7	F	1.172
AM303	15.2	M	1.168
AM288	16.0	M	3.148
AM215H	16.6	M	3.892
AM190	18.0	F	1.531
AM250	18.0	F	6.014
AM274	18.3	M	2.284
AM253	18.4	F	1.849
AM300	19.0	F	0.804
AM252	19.9	F	0.443
AM256	20.7	F	1.929
AM200	21.0	F	4.144
AM196	22.9	F	2.943
AM236	22.9	M	0.577
AM179	23.6	F	6.993
AM243	24.4	M	2.991
AM284	25.4	M	1.465
AM220	25.7	F	8.773
AM110	25.8	M	7.93
AM283	26.4	M	2.847
AM301	28.4	M	1.148
AM180	29.6	F	3.513
AM121	30.2	M	12.373

B



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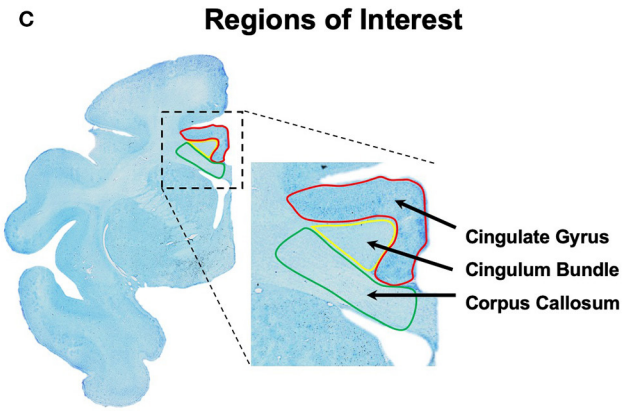

FIGURE 1 | Subjects & experimental parameters: **(A)** Table listing the 34 rhesus monkeys used in these experiments with animal ID, age, sex, and cognitive impairment score; **(B)** Linear regression of animals' age and cognitive impairment index (CII) all 34 monkeys demonstrating age-related worsening of cognitive performance; **(C)** Thionin-stained section from animal AM301 showing the regions of interest used in these experiments with the cingulate gyrus (red), cingulum bundle (yellow), and corpus callosum (green). AM, aging monkey; CII, cognitive impairment index.

of a reference group of 29 young, healthy, male monkeys. This constitutes a cognitive impairment index (CII) for each monkey as a representation of their overall learning and memory capacity. Thus, a CII (*z*-score) of <1.0 reflects no impairment, 1.0–2.0 reflects a mild impairment, while scores above 2.0 are considered severe cognitive impairment (13). The cognitive impairment distribution for the 34 rhesus monkeys examined in this study is presented in **Figure 1B**.

Brain Tissue Harvest and Processing

Upon completion of behavioral testing, brains were harvested for tissue processing and molecular experiments. Detailed descriptions of tissue collection, storage, and preparations are in (59, 60). Briefly, monkeys were euthanized by exsanguination during a two-stage transcardial perfusion of the brain that begins with Krebs buffer at 4°C to clear vasculature and cool the brain to slow proteolysis while allowing fresh tissue dissection from one hemisphere for molecular experiments. Once fresh tissue collection is complete, perfusate is switched to 4% paraformaldehyde (37°C) to ensure full fixation of the intact hemisphere. The brain is then blocked *in situ* in the coronal stereotactic plane and stored overnight in 4% paraformaldehyde at 4°C. The fixed brain tissue (including an entirely intact hemisphere) are then removed from fixative, rinsed, cryoprotected in buffer with glycerol (61), flash frozen and stored at −80°C until cut into 10 interrupted series of 30 μm-thick sections so that sections in a series are spaced at 300 μm. Cut sections were stored in buffer with 15% glycerol at −80°C until removed as a group for batch processing, a process that does not affect immunohistochemistry (59).

Immunohistochemistry

To identify $CD3^+$ T cells, tissue sections from all relevant cases were selected to obtain 6–9 sections, each spaced 2,400 μm apart, per animal to analyze the cingulum bundle, anterior body and rostrum of the corpus callosum, and cingulate gyrus (**Figure 1C**). These regions were chosen for their known age-related myelin damage in humans and our monkey model of normal aging (10, 19). The gray matter of the cingulate gyrus was chosen as a neighboring control region unaffected by normal white matter

aging. Tissue from all cases was removed from the freezer, thawed at room temperature and batch processed in the same reagents at the same time to eliminate processing variability. Sections were rinsed in 0.05 M Tris-buffered saline (TBS) at pH 7.6 to remove glycerol. To break cross-links formed during fixation, antigen retrieval was then performed by incubating tissue in Tris-EDTA buffer at pH 9 in a microwave tissue processor (PELCO Biowave, Ted Pella, Inc. Redding, CA) for 10 min at 40°C and 550 W power followed by incubation in the same buffer at room temperature for 1 h. Sections were then washed with TBS (3 × 5 min) and blocked for 1 h in SuperBlock (Life Technologies, Grand Island, NY) at room temperature followed by incubation in CD3 primary antibody (BioRad, monoclonal rat anti-CD3, clone CD3-12, MCA1477) diluted to 1:500 in TBS with 0.5% SuperBlock and 0.3% Triton X-100 (Sigma) for 24 h at room temperature on a rocker. Control sections were incubated in the same solution lacking the primary antibody. After incubation, tissue was washed in TBS (3 × 5 min) and quenched in 3% H_2O_2 for 30 min at room temperature to inactivate endogenous peroxidases. Tissue was washed again in TBS (3 × 5 min) and incubated in secondary antibody solution in TBS containing 0.5% Superblock, 0.3% Triton X, and a 1:600 concentration of biotinylated goat anti-rat secondary (Vector Laboratories, Burlingame, CA) for 1 h at room temperature. Tissue was washed in TBS (3 × 5 min) and then incubated in avidin-biotin complex using Vectastain ABC kit (Vector Laboratories, Burlingame, CA) for 1 h at room temperature. Following incubation, tissue was washed in TBS (3 × 5 min) and then incubated in a chromogen solution containing 0.5 mM 3-3-diaminobenzadine (Sigma-Aldrich, St. Louis, MO) and 0.03% hydrogen peroxide in TBS for 10 min. Tissue sections then went through a final washing in TBS (3 × 5 min) and were stored at 4°C until mounted on gelatin coated slides and air-dried for 48 h before being dehydrated through alcohols and cleared in xylenes (2 × 20 min). Slides were coverslipped with Permount (Fisher Scientific, Waltham, MA), arranged anatomically and blinded for quantification.

Fibrinogen staining was performed on 3 tissue sections spaced 7,200 μm apart per subject from a subset of the cohort to analyze the cingulum bundle, anterior body, and rostrum of the corpus callosum, and cingulate gyrus (**Figure 1C**). The same IHC protocol as described above was used, with primary antibody (DAKO, polyclonal rabbit anti-fibrinogen, A0080) at a concentration of 1:5,000 and secondary antibody (biotinylated goat anti-rabbit, Vector Laboratories, Burlingame, CA) at a concentration of 1:1,000.

To identify $LN3^+$ microglia, tissue sections were selected from a subset of the same subjects that were over the age of 20 years old to obtain 6–9 sections, each spaced 2,400 μm apart, per animal to analyze the cingulum bundle, anterior body and rostrum of the corpus callosum, and cingulate gyrus (**Figure 1C**). For IHC, sections were removed from the freezer and thawed as described above and processed through the same steps as the CD3 series except without the initial antigen retrieval step. Primary LN3 antibody (Thermo Fisher, monoclonal mouse, anti-LN3, clone LN3, MA1-35420) was used at a concentration of 1:100 and secondary was used at a concentration of 1:600 (biotinylated anti-mouse Vector Laboratories, Burlingame, CA). Color precipitation was performed with the same chromogen solution described above with the addition of 0.1 M nickel sulfate.

Multi-label immunofluorescence was performed as described above but with a 2-h blocking step, overnight incubation in primary antibodies to CD3 (1:500, BioRad, monoclonal rat anti-CD3, clone CD3-12, MCA1477), collagen IV (1:500, Sigma, monoclonal mouse anti-collagen IV, clone COL-94, C1926), and GFAP (1:1,000, Dako, polyclonal rabbit anti-GFAP, Z0334), followed by a 2-h incubation in appropriate fluorescent labeled secondary antibodies. Tissue was slide-mounted and imaged using a Zeiss LSM 710 NLO confocal microscope at high magnification (40X oil objective).

T Cell and Microglia Quantification

Unbiased stereology was performed on a Nikon E600 light microscope equipped with a Q-Imaging digital camera, a motorized stage and StereoInvestigator software (MBF Bioscience, Williston, VT). The regions of interest (cingulum bundle, corpus callosum, and cingulate gyrus) were identified and demarcated (**Figure 1C**) at low magnification (1X objective). The optical fractionator method as described previously (62) and applied to monkey tissue (60) was used to quantify CD3 and LN3 positive cells within each of these ROIs at high magnification (20X objective). Briefly, a sampling grid was placed with a randomized starting location over each outlined ROI. Within each sampling grid square, a counting frame was placed with a dissector top guard volume extending to 2 μm below the tissue section surface. Cells not intersecting exclusion planes were counted. Grid size and counting percentage was optimized to minimize the coefficient of error (CE), which is calculated as previously reported (63). CE values were $\leq$0.10 in all ROIs except the cingulate gyrus gray matter and for other ROIs in young animals. In those cases, CE values below 0.1 were unattainable due to low total number of countable T cells. The Cavalieri estimator was used to calculate the volume of each ROI. As they were counted, $CD3^+$ T cells were classified as "parenchymal" if they did not border a blood vessel. "Perivascular" T cells were classified as those surrounding a vessel (**Figures 2A–C**). $LN3^+$ microglia were identified as ramified, hypertrophic, or amoeboid based on their morphology, as previously described (64).

Fibrinogen Quantification

The same microscope setup described above was used to first outline the regions of interest (cingulum bundle, corpus callosum, and cingulate gyrus) at low magnification (1X objective). A grid was placed over each ROI so that systematic random sampling could be performed to acquire images for fibrinogen stain analysis. An image was collected at high magnification (20X objective lens) at every 6th grid site for the cingulum bundle, every 8th for the corpus callosum, and every 15th for the cingulate gyrus. Image J software (NIH) was used to convert images to 32-bit and auto-thresholded ("Default" setting) to then calculate percent area stained. Measurements within the same region of the same animal were averaged to obtain a single value representing the average percent area stained per region per subject.

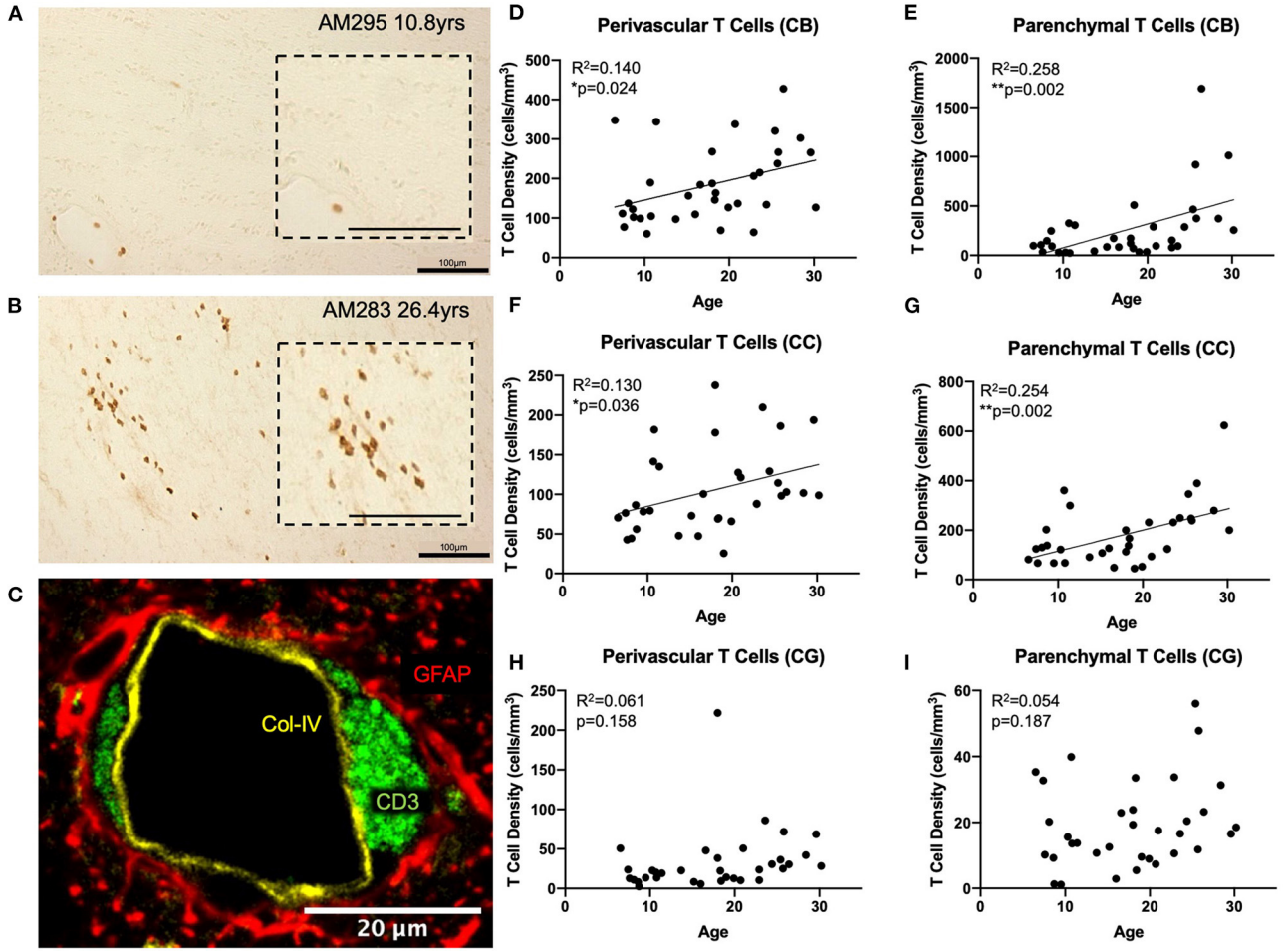

FIGURE 2 | T cells infiltrate the white matter parenchyma in aging: **(A)** $CD3^+$ IHC staining (brown) in the white matter of a young and an old **(B)** monkey illustrating the increase in density of parenchymal T cells in the aged brain. Insets show an enlarged view; **(C)** Fluorescent multi-labeling to show that perivascular T cells ($CD3^+$ in green) reside in the glymphatic space between the basement membrane (collagen IV in yellow) and the astrocytic endfeet (GFAP in red). **(D,E)** both perivascular and parenchymal T cells significantly increase in the cingulum bundle with age; **(F,G)** Perivascular and parenchymal T cells significantly increase in the corpus callosum with age; **(H,I)** T cells do not increase in the gray matter of the cingulate gyrus with age. CB, cingulum bundle; CC, corpus callosum; CG, cingulate gyrus. $N = 34$.

Statistics

All variables including ages are treated as continuous numerical values. All regression analyses were performed on independent measures using Prism with an alpha significance value of 0.05.

RESULTS

T Cells Infiltrate the White Matter Parenchyma of the Aged Brain

We used anti-CD3 to label all T cell subtypes in the CNS. Initial observation of tissue sections stained with CD3 antibody revealed the expected presence of CD3 positive T cells in the choroid plexus, ependyma lining the ventricles, and meninges as previously reported (49, 65, 66). However, it was also noted that T cells were present surrounding the penetrating vasculature as well as within the white matter parenchyma, primarily in the aged brain (**Figures 2A,B**). We designated these populations as perivascular and parenchymal, respectively.

To confirm the spatial relationship of perivascular T cells to the vasculature, we performed multi-label immunofluorescence using antibodies against CD3 (T cells), collagen IV (basement membrane of endothelium), and GFAP (glial fibrillary acidic protein—to label astrocytic endfeet around blood vessels) and found that these perivascular T cells are located in the "glymphatic space" (67), proximal to the astrocyte investment but outside the basement membrane so that they are restricted from direct contact with the brain parenchyma (**Figure 2C**). In contrast, parenchymal T cells were not associated with any vascular elements but instead were located within the brain tissue. Quantification distinguished these as unique due to their localization within the white matter.

Tissue stained for CD3 from 34 rhesus monkeys (16 male and 18 female) ages 5–30 years old (see **Figure 1A**) was analyzed to quantify parenchymal and perivascular T cell density in three regions of interest—the white matter of the cingulum bundle and corpus callosum, and the gray matter of the cingulate gyrus. As shown in **Figures 2D–G**, perivascular T cell density significantly increases with age in the cingulum bundle [$F_{(1, 32)} = 5.203$, $R^2 = 0.1399$, $p \leq 0.05$] and the corpus callosum [$F_{(1, 32)} = 4.79$, $R^2 = 0.1302$, $p \leq 0.05$]. Parenchymal T cell density also significantly increases with age in the cingulum bundle [$F_{(1, 32)} = 11.10$, $R^2 = 0.2575$, $p \leq 0.05$] and corpus callosum [$F_{(1, 32)} = 10.88$, $R^2 = 0.2537$, $p \leq 0.05$]. In the gray matter of the adjacent cingulate gyrus, T cells were rare in both the perivascular and parenchymal compartments and neither showed any significant difference with age [perivascular $F_{(1, 32)} = 2.092$, $R^2 = 0.06136$, $p = 0.1578$ and parenchymal $F_{(1, 32)} = 1.815$, $R^2 = 0.05368$, $p = 0.1874$] (**Figures 2H,I**).

Percentage of CNS T Cells in the Parenchyma Increases With Age

The perivascular T cells observed here could represent cells that are either entering or exiting the parenchyma (68), or behaving as resident border-zone regulators, which exert a variety of functions *via* cytokine signaling (46, 66). Because of this, we compared the percentages of T cells in the parenchyma (parenchymal/(parenchymal + perivascular)) to the percentage of T cells in the perivascular space across age in the cingulum bundle and corpus callosum (**Figures 3A,B**). As shown in **Figures 3C,D**, we found that in the cingulum bundle the percentage of T cells in the perivascular space was reduced while there was a complementary increase in the percentage of T cell within the parenchyma [$F_{(1, 32)} = 9.86$, $R^2 = 0.236$, $p \leq 0.05$]. This suggests an enhanced parenchymal accumulation, which could be due to increased entry, decreased egress, or a combination of both. In contrast, as shown in **Figures 3E,F**, in the corpus callosum there was no significant shift with age in the percentage of T cells in the perivascular space compared to the parenchyma [$F_{(1, 32)} = 1.11$, $R^2 = 0.0336$, $p = 0.2991$].

T Cell Infiltration Into the Cingulum Bundle Correlates With Microglial Reactivity

We assessed whether T cells were leaking into the brain parenchyma through a damaged blood brain barrier by performing immunohistochemistry to quantify fibrinogen in the brain parenchyma. Fibrinogen is a large (340 kDa) coagulation protein found in blood serum that should not be present in the brain if BBB is intact, allowing immunohistochemical staining and quantification of fibrinogen in the brain to be used to assess BBB leakiness for molecules smaller than 340 kDa (69). Fibrinogen staining in a subset of 15 monkeys (8 male and 7 female) aged 6.5–29.6 years old was quantified as percent area stained in the cingulum bundle, corpus callosum, and cingulate gyrus. In the gray matter of the cingulate gyrus staining was almost exclusively associated with the vasculature. White matter staining varied a bit between subjects and ranged from predominantly vascular associated to some glial staining of fibrinogen because it binds to CD11b receptors (70–72) (**Figures 4A,B**). As shown in **Figures 4C–E**, the percent area of fibrinogen staining in the brain parenchyma was minimal, ranging from 0.05 up to 1.7% across all ROIs and cases, confirming previous reports in a study of AD brains of fibrinogen staining in age-matched healthy controls reflecting BBB integrity (73). Importantly, staining did not change with age in the cingulum bundle [$F_{(1, 13)} = 0.00178$, $R^2 = 0.000137$, $p = 0.9670$], corpus callosum [$F_{(1, 13)} = 0.531$, $R^2 = 0.0392$, $p = 0.4791$], or cingulate gyrus [$F_{(1, 13)} = 0.000166$, $R^2 = 1.28\text{e-}005$, $p = 0.9899$]. Since T cells are much larger (5–7 μm) than fibrinogen (340 kDa), it was unlikely that age-related T cell infiltration was due to breakdown of the BBB. Nevertheless, as a test of whether T cells might be associated with this low level of fibrinogen, we examined the relationship of fibrinogen levels to perivascular and parenchymal T cell density. As shown in **Figures 5A–D**, there was no correlation between T cell infiltration and the amount of fibrinogen in the cingulum bundle [perivascular $F_{(1, 13)} = 0.0409$, $R^2 = 0.00313$, $p = 0.8429$ parenchymal $F_{(1, 13)} = 0.0643$, $R^2 = 0.00492$, $p = 0.8038$] or the corpus callosum [perivascular $F_{(1, 13)} = 0.222$, $R^2 = 0.0168$, $p = 0.6454$, parenchymal $F_{(1, 13)} = 1.44$, $R^2 = 0.0999$, $p = 0.2512$], suggesting that T cell infiltration does not occur across a leaky BBB in these monkeys.

We next examined whether T cell entry into the brain may be in response to increases in microglial reactivity. To assess whether the observed age-related increase in microglial reactivity in the oldest subjects of this cohort (≥20 years old, $n = 13$) might be related to infiltrating T cells, we stained tissue of the same section spacing as CD3 with the LN3 antibody (anti-MHC-II receptor) (**Figures 6A,B**). We were specifically curious about microglia that are involved in antigen presentation *via* MHC-II receptors, which have been suggested to stimulate and activate CNS T cells (74). As described by Karperien et al. (64) we classified LN3 positive microglial subtypes by their morphology as ramified, hypertrophic, or amoeboid microglia reflecting the least to most inflammatory/phagocytic phenotypes (**Figure 6C**). Quantifying microglia by this classification scheme we found in the cingulum bundle age-related increases in density that approached significance for both amoeboid [$F_{(1, 11)} = 3.36$, $R^2 = 0.234$, $p = 0.0941$] and hypertrophic [$F_{(1, 11)} = 2.63$, $R^2 = 0.193$, $p = 0.1330$] microglia (**Figures 6D,E**). There was a significant decrease in the density of ramified microglia with age [$F_{(1, 11)} = 5.27$, $R^2 = 0.324$, $p \leq 0.05$] (**Figure 6F**). These results are aligned with previous findings in our model that $LN3^+$ amoeboid microglia increase in the cingulum bundle with age (35). We then examined whether this increased microglial reactivity is related to T cell infiltration and found that an increase in amoeboid microglial density significantly correlated with higher density of parenchymal T cells [$F_{(1, 11)} = 16.2$, $R^2 = 0.595$, $p \leq 0.05$] (**Figure 6G**). There was no relationship between hypertrophic [$F_{(1, 11)} = 0.224$, $R^2 = 0.0199$, $p = 0.6455$) or ramified [$F_{(1, 11)} = 1.58$, $R^2 = 0.126$, $p = 0.2346$) microglia density and T cell density (**Figures 6H,I**). These data suggest that T cell infiltration is associated with increased neuroinflammation as quantified by density of pro-inflammatory microglia.

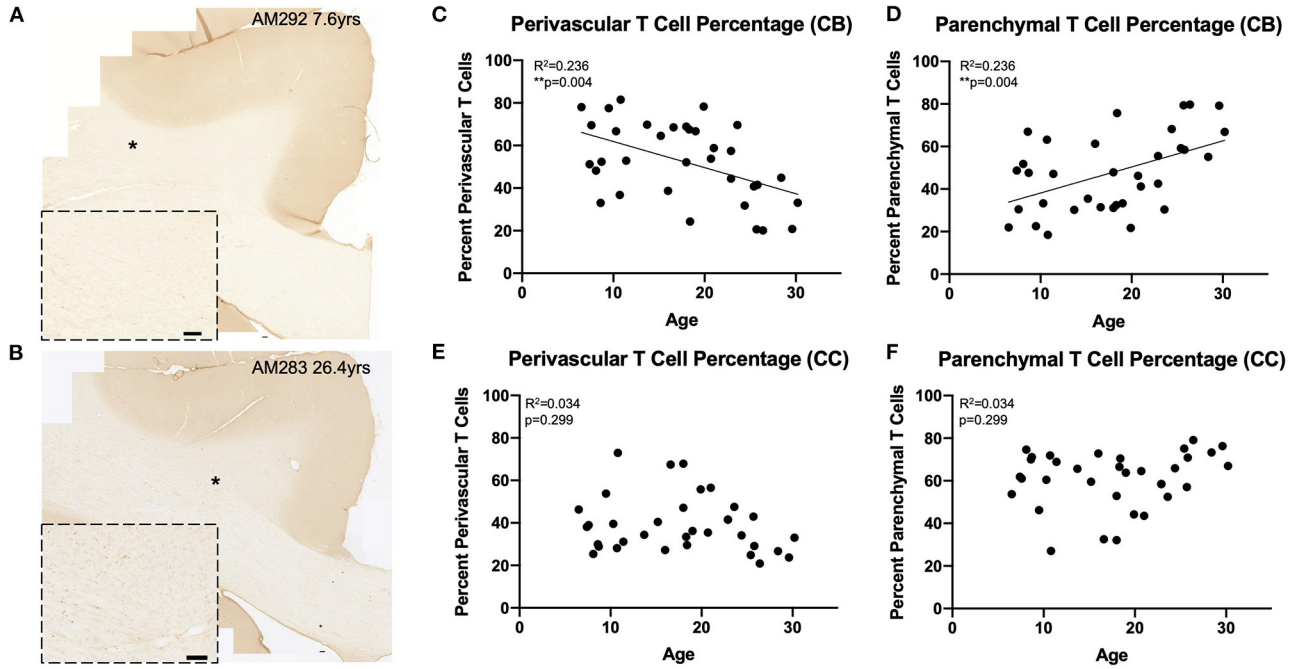

FIGURE 3 | Greater proportion of T cells in the white matter parenchyma with age: **(A,B)** Representative region of interest images for $CD3^+$ stained T cells in a young **(A)** and old **(B)** monkey. Inset shows detail, location of inset marked with * in large image. Scale bars are 100 μm. **(C,D)** The percentage of perivascular T cells decreases while the percentage in the parenchyma increases with age in the cingulum bundle; **(E,F)** The percentage of perivascular T cells as well as those in the parenchyma does not change with age in the corpus callosum. CB, cingulum bundle; CC, corpus callosum. $N = 34$.

Higher Percentage of Parenchymal T Cells Correlates With Cognitive Impairment

We compared our findings of T cell density to the cognitive scores established for each subject as previously described (**Figure 1B**). As shown in **Figures 7A,B**, there was no significant relationship between absolute density of parenchymal T cells and the CII in the cingulum bundle [$F_{(1, 32)} = 2.02$, $R^2 = 0.0595$, $p = 0.1646$] or in the corpus callosum [$F_{(1, 32)} = 1.63$, $R^2 = 0.0484$, $p = 0.2113$]. In contrast, when T cell accumulation in the parenchyma was assessed by percentage parenchymal T cells as described above—parenchymal/(parenchymal + perivascular)—there was a significant relationship between the percentage of T cells in the parenchyma of the cingulum bundle and cognitive impairment (CII) [$F_{(1, 32)} = 4.86$, $R^2 = 0.132$, $p \leq 0.05$] but not in the corpus callosum [$F_{(1, 32)} = 0.0132$, $R^2 = 0.000413$, $p = 0.9092$] (**Figures 7C,D**). This is in agreement with previous studies showing that neither white matter damage nor neuroinflammation in the corpus callosum are predictive of age-related cognitive decline in the rhesus monkey even when these changes in the cingulum bundle are (19, 35).

DISCUSSION

Summary of Results

Primate and human aging are characterized by varying degrees of cognitive decline caused by an accumulation of damaged myelin sheaths rather than neuronal degeneration like that observed in AD (3–7, 19, 75). Myelin sheath pathology has been previously characterized in the frontal white matter of our primate model of normal cognitive aging (19, 23, 76, 77). Closer examination of these regions revealed a role of activated, dystrophic microglia that correlated with the severity of cognitive impairment (35). Here we set out to examine whether T cells may be responding to increased neuroinflammation and myelin damage in brain regions known to be vulnerable to age-related myelin pathology in the normal aging rhesus monkey. The main findings of this study are: (1) In normal aging, T cells that are normally excluded from the brain in healthy young adults, increase in the perivascular space surrounding cortical blood vessels and infiltrate into the parenchyma of the white matter but not the gray matter. (2) In the white matter of the cingulum bundle but not the adjacent corpus callosum, the percentage of CNS T cells that infiltrate and accumulate in the white matter parenchyma increases with age compared to those in the perivascular space. (3) T cells do not passively "leak" into the brain through a damaged BBB but instead infiltrate in a manner that correlates with proinflammatory microglia classified as amoeboid (phagocytic), suggesting an active process. (4) Most importantly, the percentage of parenchymal T cells in the cingulum bundle correlated with age-related impairments in cognitive function. These data suggest that T cells in the white matter may directly contribute to age-related white matter damage and cognitive decline in the healthy, aged brain. Hence,

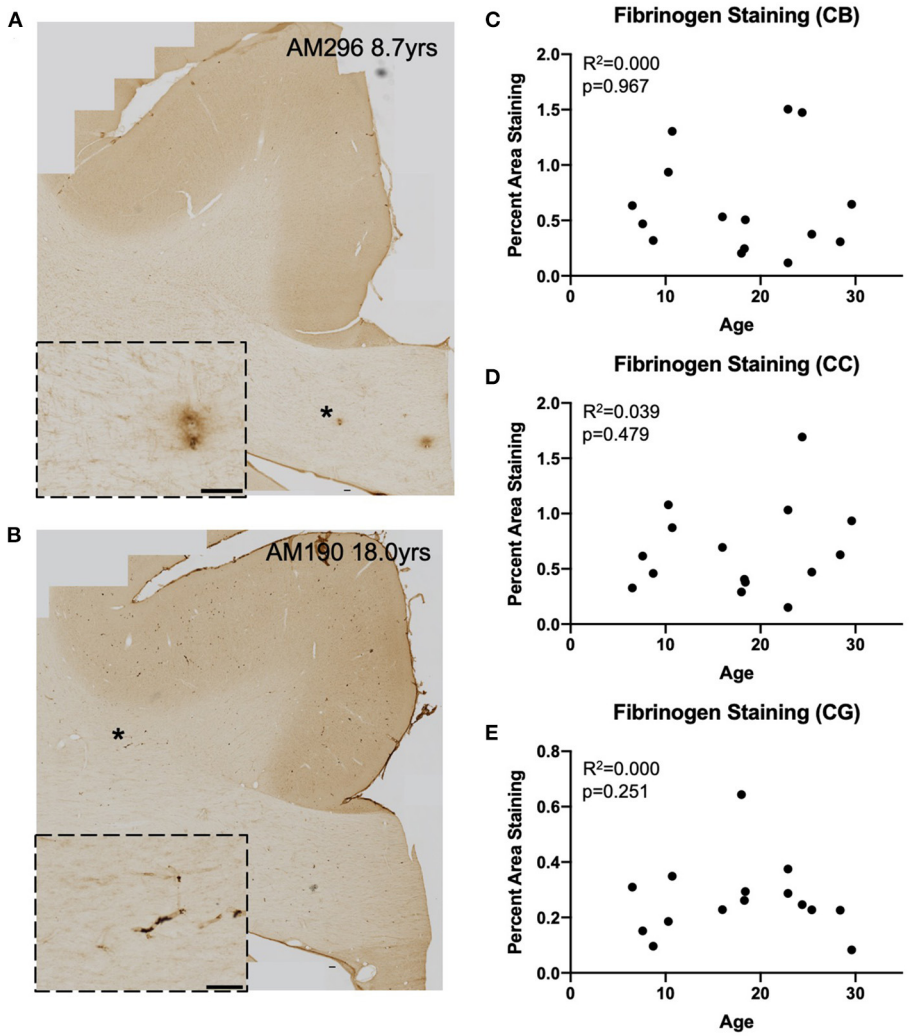

FIGURE 4 | Fibrinogen staining with age: IHC for fibrinogen was used to assess possible leakage from the vasculature in a representative subset ($n = 15$) of monkeys **(A,B)** Representative images to exhibit the variability in fibrinogen staining between subjects. Insets show detail of vascular and glial staining that were quantified as positive signal. Location of inset marked with * in large image. Scale bars are 100 μm. **(C–E)** There is an absence of significant age-related fibrinogen staining as percent are in the cingulum bundle **(C)**, the corpus callosum **(D)**, and the gray matter of the cingulate gyrus **(E)**. CB, cingulum bundle; CC, corpus callosum; CG, cingulate gyrus.

T cells infiltrating the CNS parenchyma may contribute to white matter damage making them a target for future therapeutic interventions aimed at ameliorating cognitive aging.

Cognitive Impairment and Neuroinflammation in Normal Aging

Cognitive decline in normal, non-neurodegenerative aging, occurs in monkeys (23) and humans (3, 7). Careful studies that exclude early stage Alzheimer's cases show that these age-related cognitive impairments are not the result of the degeneration or loss of cortical neurons but likely result, at least in part, from an age-related accumulation of myelin sheath damage followed by subsequent demyelination and axon loss producing a cortical "disconnection" (19, 21, 24). The processes leading to myelin damage and the failure of myelin sheath repair in normal aging are not clearly understood, but it has been suggested that oxidative damage and inflammation contribute to damage of myelin lipids and oligodendrocyte senescence (78–80). In normal aging monkeys, an age-related increase myelin debris could overburden the phagocytic capacity of microglia leading to phagocytic inefficiency and accumulation of interstitial myelin debris. This in turn can impair maturation of oligodendrocyte precursor cells into mature, myelinating oligodendrocytes, reducing their ability to repair age-related myelin sheath damage (26–28, 31, 32, 34, 81). Moreover, there is a plethora of data demonstrating a generalized, age-related increase in markers of inflammation both in the periphery and within the CNS, often referred to as "inflammaging" (82, 83).

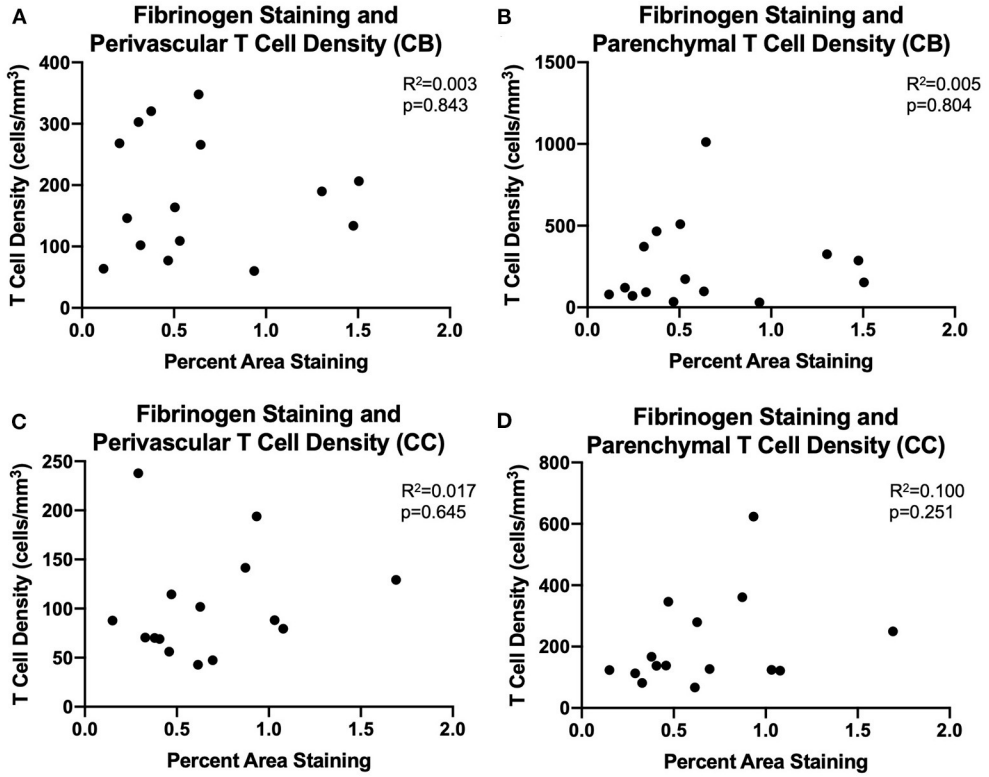

FIGURE 5 | BBB leakiness does not account for T cell infiltration into the white matter: **(A–D)** Fibrinogen presence measured by percent area staining did not correlate with perivascular or parenchymal T cell density in the cingulum bundle or the corpus callosum. CB, cingulum bundle; CC, corpus callosum. $N = 15$.

Neuroinflammation is known to contribute to many age-related neurodegenerative diseases as well as normal age-related white matter damage (34, 82). The density of LN3 positive activated and Gal-3 positive phagocytic microglia increase specifically in frontal white matter tracts with age and correlate with cognitive impairment (19, 35). Further, in the aging brain, microglia appear to be chronically pro-inflammatory, with increased expression of IL-1β, IL-6, and TNF-α and decreased expression of anti-inflammatory cytokines such as IL-10 (36). In the periphery, secretion of pro-inflammatory cytokines by macrophages can lead to recruitment and activation of T cells (84). Here, we have confirmed an age-related increase in the pro-inflammatory phenotype of microglia within the white matter and further demonstrate a positive relationship between age-related increased inflammatory microglia and increased density of T cells within the white matter parenchyma.

In addition to clearance by microglial phagocytosis, myelin debris has been found to be cleared from the brain by the CSF where it may drain through the lymphatics into the deep cervical lymph nodes (67, 85, 86). Once myelin debris enters the peripheral circulation, it can interact with antigen presenting cells (APCs) in the lymph nodes. This process has been observed in many demyelinating diseases and corresponding animal models (87). Of note, myelin-reactive T cells have been observed in the periphery of healthy individuals lacking gross myelin pathology (85). With age, myelin basic protein increases in the CSF of our aged monkeys (unpublished data), which would enable increased antigen exposure to the adaptive immune system and could explain why T cells may become myelin reactive and traffic to specific white matter regions in aging brain, though this remains to be confirmed in our aging monkeys.

T Cell Trafficking Into the CNS

In Alzheimer's and other neurodegenerative diseases, there is a breakdown of blood brain barrier integrity, which may, at least in part, account for the T cell infiltration reported in human brain (73, 88–90). Here, we show that in the normal aging monkey brain, the minimal fibrinogen extravasation that we do observe is not associated with T cells. Hence, T cell infiltration into the brain in normal aging seems likely to be an active and regulated process and not a result of leakage through a damaged BBB.

The trafficking of T cells out of the vasculature and into inflamed tissues in the periphery relies on a process called "diapedesis," which involves stepwise expression of receptors and ligands on endothelial cells (e.g., E- and P-selectins) and T cells (e.g., LFA-1) to facilitate slowing, rolling, adhesion and migration of T cells across the endothelium. Slowing and rolling are mediated by endothelial expression of selectins (E- and P-selectins), followed by their expression of integrins (ICAM and

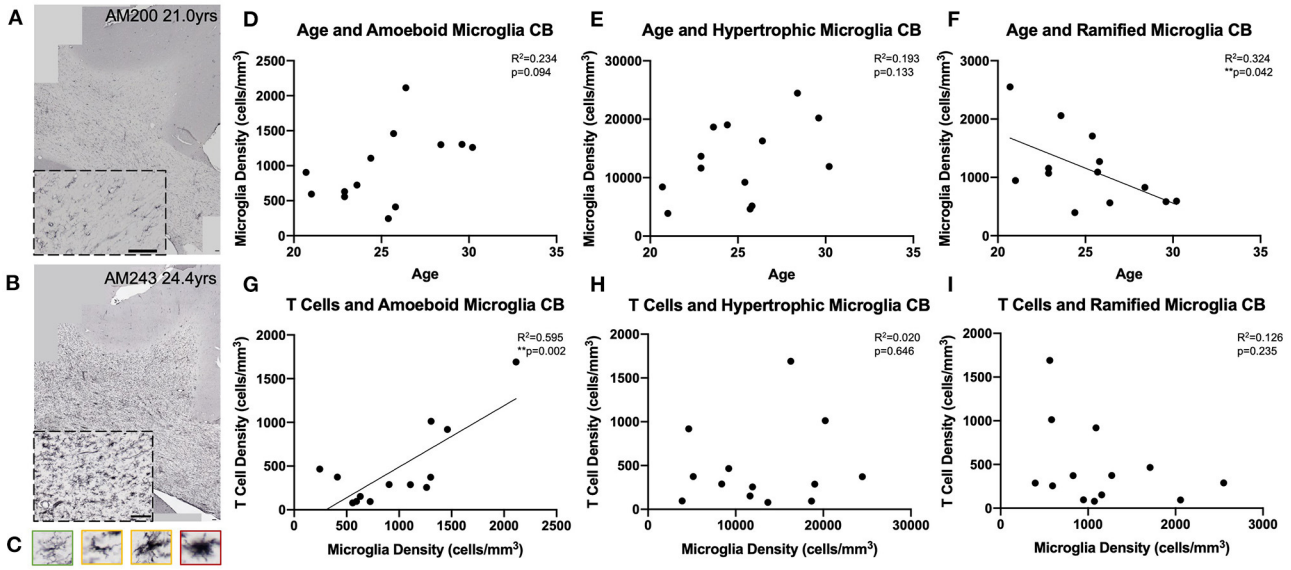

FIGURE 6 | Microglial reactivity correlates with T cell infiltration in the cingulum bundle: **(A,B)** Representative region of interest images depicting lower **(A)** and higher **(B)** levels of microglial reactivity with LN3 staining. **(C)** Microglial reactivity in the cingulum bundle was quantified by counting the density of $LN3^{+}$ stained cells by morphology, grouping them into ramified (green outline), hypertrophic (yellow outline), and amoeboid (red outline). The density of amoeboid **(D)** and hypertrophic **(E)** microglia did not change with age, however there was a significant decline in the density of ramified microglia with age **(F)**. The density of amoeboid microglia correlated with the density of T cells that infiltrated the cingulum bundle parenchyma **(G)**. T Cell density did not correlated with hypertrophic **(H)** or ramified **(I)** microglia density. CB, cingulum bundle. $N = 13$.

VCAM) (87, 91). Mechanisms of T cell entry into the CNS parenchyma are under debate, as it does not appear to follow the same sequence of steps observed in peripheral tissues and thus could serve to protect the brain's immune privilege status (68). One example of how the CNS protects its immune privilege status, is that astrocytes secrete factors that down-regulate the expression of E- and P-selectin on the BBB endothelium (92). Interestingly, in murine models of MS (i.e., EAE), it has been shown that T cell entry into the CNS is not dependent on E- and P-selectins as it is in humans but instead appears to rely exclusively on ICAM and VCAM expression on endothelial cells (92). Conversely, in the non-inflamed CNS, entry of T cells across the murine BBB seems to depend on E- and P-selectins and *not* ICAM or VCAM (93). As such, these differences among species has made it difficult to study concretely how T cells enter the CNS in health and disease.

For the non-inflamed CNS, the majority of studies agree that T cells can cross into the CSF across an intact BBB *via* the choroid plexus and/or the leptomeningeal blood vessels for surveillance (66, 87, 94). Yet, other studies have suggested migration can occur across the penetrating parenchymal vessels (95). To complicate the story further, studies have shown that E- and P-selectins are not expressed by the endothelium of the penetrating vessels in the human brain, rather this is exclusive to the endothelium of the meningeal blood vessels and those of the choroid plexus (96). Consequently, it is not possible to draw firm conclusions as to the directionality (entry or exit) of the perivascular T cells surrounding the penetrating parenchymal vessels in the aging monkey brain. However, we have shown an increase in the percentage of T cells within the parenchyma, which could suggest either increased entry, decreased exit, or a combination of both. Nevertheless, since this buildup of parenchymal T cells correlates with worsening cognitive performance, it is essential to obtain a greater understanding of trafficking mechanisms as this may provide avenues to reduce the density of CNS parenchymal T cells and prove beneficial to age-related cognitive decline.

T Cells in the Diseased CNS

T cells residing at the choroid plexus are able to surveil the CSF and can cross the BBB upon antigen recognition in cases of injury or disease (66, 97). Following traumatic brain injury, microglia recruit first the innate immune system followed by cells of the adaptive immune system, including T cells, to the affected area where they participate in the local CNS immune response aimed at neurorestoration (98). Using an optic nerve crush injury model in rodents, researchers demonstrated that CNS-specific T cells (e.g., myelin recognizing) reduced neuronal loss (99). CNS-antigen recognizing T cells have often been considered largely detrimental due to their role in autoimmune diseases like MS (100). However, a more beneficial role of CNS auto-reactive cells has been termed "protective autoimmunity," which protects neurons from secondary degeneration known to occur after initial injury (101). This protective role depends on a population of CNS antigen-specific memory T cells that reside in the meninges and choroid plexus and are primed to respond to damage following injury and promote beneficial neuroinflammation and lymphocyte recruitment for neuroprotection and repair (66, 88).

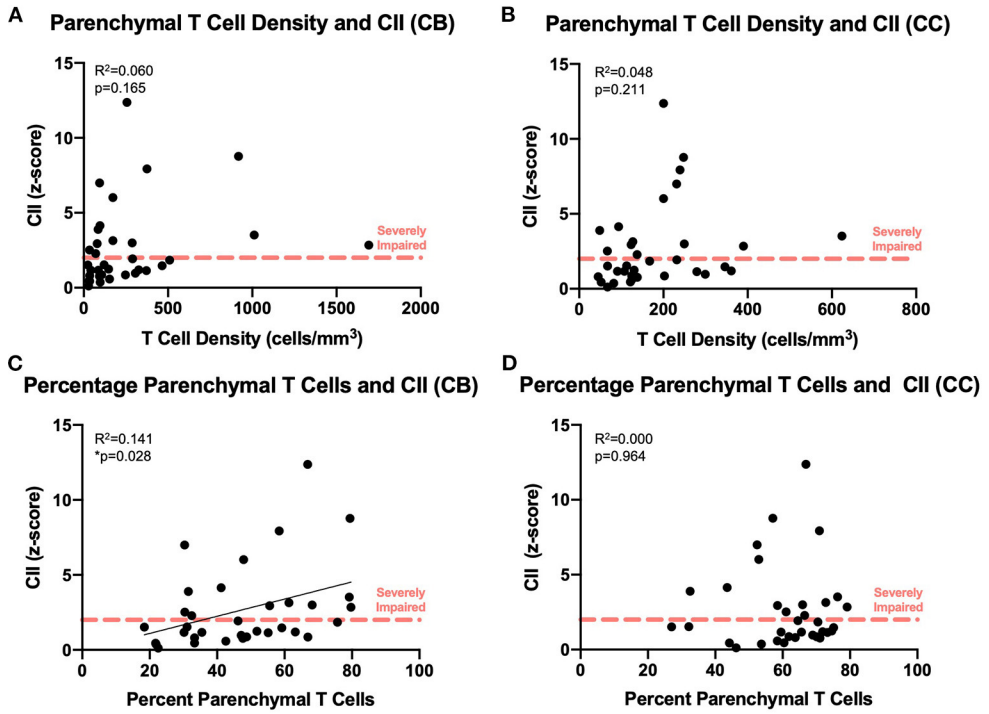

FIGURE 7 | Increased percentage of parenchymal T cells in the cingulum bundle correlates with worsening cognitive performance: The density of parenchymal T cells does not correlate with cognitive impairment in the cingulum bundle **(A)** or the corpus callosum **(B)**; the percentage of T cells in the parenchyma correlates with the cognitive impairment index in the cingulum bundle **(C)** but not in the corpus callosum **(D)**. CB, cingulum bundle; CC, corpus callosum; CII, cognitive impairment index. $N = 34$.

In Alzheimer's Disease, neuroinflammatory processes have been examined predominantly by looking at reactive astrocytes and microglia (102–104). Though early reports suggested the presence of T lymphocytes in diseased patient brains (105, 106), their role in AD has only been recently explored. It has been shown that AD plaques are surrounded by activated microglia that are likely interacting with $CD8^+$ lymphocytes and might play a role in plaque pathology (107). Further, these AD-associated $CD8^+$ T cells may contribute to synaptic plasticity dysfunction and preventing their infiltration results in beneficial restoration of neural plasticity gene expression programs (108). Characterization of T cells circulating in the CSF of patients with AD has revealed clonal expansion of $CD8^+$ effector T cells with potential implications for neurodegeneration *via* their cytotoxic effector functions (109). Our data confirm these findings of age-related infiltrating T cells but we further characterize their infiltration specifically into white matter regions with known myelin pathology and correlate this influx of T cells with normal cognitive decline.

Much of our understanding of T cells' interactions with myelin comes from studies in human multiple sclerosis and corresponding animal models. These studies have shown that exposure of myelin epitopes to peripheral T cells may lead to auto-attack and demyelination as T cells initially sensitized in the periphery become reactivated upon entry into the CNS (110–112). Studies of MS therapeutics aimed at preventing T cell entry into the CNS uncovered a previously under-appreciated role of T cell surveillance for viruses and showed that blockade of T cells was potentially lethal (113). In the case of viral infection, such as JC virus, T cells specific to the virus are able to detect the presence of infection *via* immunosurveillance of the CSF and mount an immune response to prevent progression into the fatal demyelinating disease Progressive Multifocal Leukoencephalopathy (PML) [review e.g., (114)]. During healthy brain surveillance, T cells downregulate their cytolytic granzyme and pro-inflammatory cytokine expression thus preventing autoimmune demyelination (66, 115, 116). It has been suggested that age-related exacerbation of neuroinflammation may disrupt these normal homeostatic functions of immunity and immune surveillance (82).

T Cells in the Healthy CNS

Recently it has been reported that T cells are beneficial to cognition, including learning and memory (46, 117). Immunodeficient mice, with depleted T cells, display impaired learning and memory which can be rescued by restoration of a normal functioning T cell repertoire (118). These effects are believed to be mediated by $CD4^+$ T cells that secrete IL-4, which induces astrocytes to secrete BDNF (119). Myelin-reactive T cells have been shown to play a role in hippocampal neurogenesis and supporting spatial learning (117). However, age-related shifts in the immune system, known as "inflammaging," cause choroidal

T cells to shift toward a pro-inflammatory profile that may be detrimental to cognition (49). Further, T cell infiltration into the temporal lobe of aged mice has been reported to inhibit hippocampal neurogenesis and lead to impairment of spatial learning and memory (51). Recent studies of human AD have shown an influx of $CD8^+$ T cells in age-matched control brains, but have not extensively characterized the white matter across the entire lifespan, which we have done here (108).

Our current study did not examine the hippocampus or choroid plexus but instead focused on white matter regions that have previously been associated with both myelin pathology and with cognitive decline observed in normal aging monkeys (19) and humans (75). We specifically were interested in following up on our previous findings that white matter damage and microglial dysfunction in the cingulum bundle of the rhesus monkey were associated with age-related cognitive impairment (19, 35) but now add the observation that T cell infiltration correlates with regional neuroinflammation (activated microglia) as well as cognitive decline.

Very few studies systematically examine the white matter in normal aging so little is known of the function of parenchymal infiltrating T cells. Rodent studies of white matter aging are often inadequate at characterizing the brain late enough in aging to detect the white matter changes we observe in humans and primates (66, 120). Hill et al. found myelin damage comparable to monkeys and humans only in extremely old mice >600 days or 20 months (120). Several reports that examined the normal aging mouse brain have reported an age-related increase in T cells but many report that they are mostly perivascular, meningeal, or choroidal-associated (66, 121). Ritzel and colleagues studied mice out to 22-months of age and report these age-associated T cells promote downregulation of microglial reactivity and encourage a state of surveillance (66). Instead, we suggest T cells that actively infiltrate aging white matter parenchyma may be myelin reactive and could play a detrimental role in allowing or perhaps exacerbating age-related myelin damage and associated cognitive decline. While we have confirmed the age-related increase in perivascular T cells, which may indeed be regulatory, we add that in the aging primate, specifically those exhibiting the most severe cognitive decline, T cells increase in the parenchyma as well. Of note, it is possible that the myelin sheath damage, accumulation of myelin debris, and microglial dysfunction that we observe in our monkeys (19, 35) lead to a more detrimental T cell response in the aging white matter parenchyma of the primate brain, which has a significantly greater proportion of white matter tissue than rodents (122).

Conclusions

The observations reported here show that T cells infiltrate the aged brain white matter in the absence of infection, damage or blood brain barrier breakdown. Moreover, this infiltration is associated with age-related cognitive impairment. This T cell infiltration into the aging white matter parenchyma constitutes a novel observation that may reflect the mechanism by which cells of the adaptive immune system, during normal aging, contribute to neuroinflammation, white matter pathology and ensuing cognitive impairments. The present data show that in the normal aging primate, T cell infiltration of the brain white matter is likely an active infiltration that correlates with increases in microglia with an inflammatory phenotype. We hypothesize that these infiltrating T cells may be myelin reactive, due to increased exposure to myelin antigens with age, and thus could directly exacerbate myelin pathology in aging. These memory T cells may become activated by the pro-inflammatory neuro-environment (123) and secrete granzymes that may damage the myelin sheath (124). Alternatively, infiltrating T cells may be regulatory and enter in an attempt to moderate neuroinflammation by downregulating microglial reactivity (66). However, given our findings that T cell infiltration is significantly associated with worsening cognitive impairment, we believe that T cells are likely to play a detrimental role in the propagation of age-related neuroinflammation and myelin damage. We suggest that this may constitute a model on which to design more mechanistic studies to examine trafficking routes, antigen-specificity, and inflammatory phenotype of T cells that infiltrate the aging primate brain. Hence these infiltrating T cells offer a new target for interventions aimed at slowing or even reversing age-related neuroinflammation and cognitive decline.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Boston University Institutional Animal Care and Use Committee (IACUC).

AUTHOR CONTRIBUTIONS

Experimental design, execution, and data analysis by KB and DR. Fibrinogen experiments largely carried out by PC. Behavioral data collection and management by TM. Manuscript preparation and editing by all.

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Common Peripheral Immunity Mechanisms in Multiple Sclerosis and Alzheimer's Disease

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Neurodegenerative diseases are closely related to inflammatory and autoimmune events, suggesting that the dysregulation of the immune system is a key pathological factor. Both multiple sclerosis (MS) and Alzheimer's disease (AD) are characterized by infiltrating immune cells, activated microglia, astrocyte proliferation, and neuronal damage. Moreover, MS and AD share a common pro-inflammatory signature, characterized by peripheral leukocyte activation and transmigration to the central nervous system (CNS). MS and AD are both characterized by the accumulation of activated neutrophils in the blood, leading to progressive impairment of the blood–brain barrier. Having migrated to the CNS during the early phases of MS and AD, neutrophils promote local inflammation that contributes to pathogenesis and clinical progression. The role of circulating T cells in MS is well-established, whereas the contribution of adaptive immunity to AD pathogenesis and progression is a more recent discovery. Even so, blocking the transmigration of T cells to the CNS can benefit both MS and AD patients, suggesting that common adaptive immunity mechanisms play a detrimental role in each disease. There is also growing evidence that regulatory T cells are beneficial during the initial stages of MS and AD, supporting the link between the modulatory immune compartments and these neurodegenerative disorders. The number of resting regulatory T cells declines in both diseases, indicating a common pathogenic mechanism involving the dysregulation of these cells, although their precise role in the control of neuroinflammation remains unclear. The modulation of leukocyte functions can benefit MS patients, so more insight into the role of peripheral immune cells may reveal new targets for pharmacological intervention in other neuroinflammatory and neurodegenerative diseases, including AD.

Keywords: multiple sclerosis, Alzheimer's disease, neuroinflammation, neutrophils, monocytes, T cells

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INTRODUCTION

Multiple sclerosis (MS) and Alzheimer's disease (AD) are two of the most widely studied central nervous system (CNS) pathologies. MS is the most common inflammatory neurological disease in young adults, whereas AD is a neurodegenerative disorder that occurs more frequently in the elderly population and is the most common type of dementia. The number of MS and AD patients is growing continuously, highlighting the need to find new disease mechanisms and new therapeutic

approaches (1, 2). MS and AD are both multifactorial diseases and the identification of their etiopathogenetic mechanisms is challenging. Genetic risk factors and environmental triggers are the principal risk factors for both MS and AD (3, 4).

From the neuropathological perspective, the early phases of MS, defined as relapsing-remitting MS (RRMS), are characterized by primary demyelination areas known as plaques, which are located in the white and gray matter. In contrast, the final phases (secondary progressive MS) are associated with axonopathy, neuronal death, and synaptic loss, correlating with the permanent motor disability classically shown by MS patients (5). AD neuropathology is characterized by two main hallmarks: amyloid β plaques and tau tangles. Both structures are formed from aggregated proteins, in one case due to the incorrect processing of amyloid precursor protein (APP), and in the other due to the hyperphosphorylation of tau, a microtubule-associated protein required to maintain neuronal architecture and function.

The involvement of immune and inflammatory reactions in the pathogenesis of MS has been understood for decades, but the same association was only recently identified in AD. The concept of CNS exclusion from surveillance and inflammatory responses mediated by peripheral immune cells was reconsidered more than 15 years ago describing how circulating immune cells enter into the brain for protective tissue immunosurveillance and has been recently reviewed following the discovery of the cerebral lymphatic system and its role in CNS physiology (6–8). Furthermore, strong evidence of peripheral immune cell trafficking into the CNS has been provided during the immune responses that occur during MS and its animal model, experimental autoimmune encephalomyelitis (EAE), confirming that the CNS is not an immune-privileged environment (9). More recently, immune cell trafficking has also been documented in AD and was shown to be detrimental in transgenic mice with AD-like disease (10, 11). Neuroinflammation in both MS and AD is also characterized by the activation of microglia and astrocytes, leading to the secretion of pro-inflammatory cytokines and chemokines that recruit more immune cells from the periphery to the CNS (12, 13). Interestingly, the specialized pro-resolving lipid mediators, which mediate inflammation resolution and reduce neutrophil and monocytes infiltration into the brain, are impaired in MS and AD patients and their levels correlate with disease severity (14–16). These defects in the resolution pathways further emphasize the common detrimental role of peripheral immune cells in the maintenance of neuroinflammatory process in MS and AD.

Inflammation during neurodegenerative disorders is not restricted to the CNS. Indeed, systemic inflammation has been confirmed in MS and AD, including the secretion of pro-inflammatory cytokines in peripheral domains such as the blood, cerebrospinal fluid (CSF), liver, and gut (12, 17–20). The involvement of peripheral inflammation mechanisms and immune cells in MS and AD provides strong evidence of immune dysregulation, but it is unclear whether this is a causal link in each disease or a secondary phenomenon triggered by brain injury.

Whereas, the role of humoral response has been reviewed elsewhere, here we discuss the common immune mechanisms in MS and AD and describe how neutrophils, monocytes and T cell subpopulations use similar mechanisms in MS and AD to migrate into the CNS and induce neuroinflammation and tissue damage (21). These insights suggest that interfering with shared cellular and molecular mechanisms may lead to common therapeutic approaches for MS and AD.

NEUTROPHILS: THE EMERGING PLAYERS IN MS AND AD

Neutrophils are highly reactive leukocytes with a frontline role in the maintenance of tissue homeostasis during pathological conditions, including infections and tissue damage (22). Neutrophils are highly adaptable cells due to their remarkable plasticity, and can therefore adjust their phenotype and functions in response to various environmental stimuli, triggering acute inflammatory responses (23). However, when prolonged tissue stress and damage induce sterile inflammation, neutrophils play a more subtle detrimental role, leading to chronic tissue damage that can promote pathological conditions such as autoimmunity and neurodegenerative diseases if left uncontrolled (24). These heterogeneous cells have attracted significant interest given their ability to facilitate sterile and chronic inflammation (24, 25).

Infiltrating neutrophils have been detected in the brains of MS and AD patients (10, 26). Evidence for the early involvement of neutrophils in MS includes their correlation with hyperacute lesions and altered blood-brain barrier (BBB) permeability in humans, and their involvement in the preclinical phase of EAE and acute relapses in these animal models (26, 27). Studies in AD models also suggest that neutrophils may contribute to the initial disease stages, which are characterized by increased BBB permeability, neutrophil intravascular adhesion, and invasion of the CNS (10, 11). Indeed, in animal models of both MS and AD, neutrophils accumulate in the brain before clinical manifestation, representing a major source of inflammatory mediators during early disease stages (10, 28, 29). We and others have shown that blocking neutrophil recruitment at early disease stages reduces the disease burden and tissue damage in animal models of both MS and AD (10, 30, 31). However, neutrophils continue to accumulate in the CNS throughout the disease course, suggesting these cells also play a role in disease progression and chronicity (10, 28, 29).

In MS and AD patients, the neutrophil to lymphocyte ratio (NLR) is a classical blood marker of inflammation. In MS, the NLR increases during progression and relapses, whereas in AD it correlates with cognitive impairment (32–35). In both diseases, a large proportion of circulating neutrophils is primed, as shown by the induction of activation markers such as CD11b and CD177 (36–39). Interestingly, high levels of CD11b also coincide with relapses in MS patients and correlate with the severity of the cognitive deficit in AD, suggesting that circulating neutrophils with a primed phenotype may cross the cerebral vasculature to the CNS more readily (36, 37). A similar mechanism has been proposed for CD11a/CD18 (LFA-1 or $\alpha_L\beta_2$) in the recruitment of neutrophils in AD models (10). Furthermore, peripheral hyper-activated neutrophils secrete inflammatory mediators and intravascular neutrophil extracellular traps (NETs), thus

contributing to BBB damage and disease development (10, 24, 40) (**Figure 1**, **Table 1**).

During inflammation, the constitutive expression of CXCR2 (a chemokine receptor for the ELR^+ chemokines CXCL1-3 and CXCL5-8) on mature neutrophils is strongly associated with neutrophil mobilization from the bone marrow to the bloodstream and their migration from the bloodstream to the site of injury (71). CXCL8, a CXCR2-dependent neutrophil chemoattractant, is more abundant in the plasma and CSF of MS and AD patients, and is linked to disease activity, suggesting that neutrophil migration is relevant in both diseases (44–48). Moreover, activated astrocytes produce CXCL1 (another CXCR2 ligand) at the lesion edges in EAE mice, and high levels of CXCL1, CXCL5, and CXCL8 are detected in the serum of MS patients, supporting a role for CXCR2 in the infiltration of neutrophils into the CNS in this disease (36, 44). CXCL1 is also produced by oligodendrocytes in EAE mice, attracting neutrophils into the CNS, exacerbating clinical impairment and enhancing BBB leakage (49). Interestingly, in the CNS of EAE mice, infiltrating T helper (Th) 17 cells stimulate the local release of CXCL1 and CXCL2, which leads to neutrophil recruitment (72). On the other hand, microglia in murine models of AD express CXCL1, and the levels of this chemokine in the CSF of AD patients correlate with cognitive impairment, suggesting that CXCL1 is also important in AD (50, 51) (**Figure 1**, **Table 1**). Restricting the infiltration of neutrophils using inhibitors of CXCR1 and CXCR2 has shown therapeutic efficacy in several experimental models of neuroinflammation including EAE, suggesting this may also be the case in animals with AD-like disease (73–75).

Amyloid β may also play a role in both AD and MS (76, 77). In AD brains, infiltrating neutrophils are closely associated with amyloid β deposits, and amyloid β peptides trigger the rapid integrin-dependent adhesion of neutrophils via G protein coupled receptors (10, 78). The non-random distribution of myeloperoxidase (MPO)-producing cells, presumably neutrophils, in the brain parenchyma of AD patients underlines the potential role of amyloid β as a chemoattractant that establishes a pro-inflammatory microenvironment to recruit circulating neutrophils (10). We speculate that the presence of amyloid β deposits in MS could also help to recruit neutrophils into the brain. The abundance of MPO and elastase in the blood and CNS of MS patients suggests that neutrophils may contribute to these pathological findings (56, 57). Moreover, neutrophil elastase is associated with the spread of MS lesions and clinical progression, whereas peripheral MPO activity is considered a predictor for executive function decline in AD patients (29, 46).

Neutrophil migration into the CNS during early or late phases of neuroinflammation plays a crucial role in BBB impairment. During migration, "outside-in" signaling generated following selectin and integrin engagement can induce ROS production by direct NADPH oxidase complex activation and release of other inflammation mediators such as cytokines (79–83). Moreover, it has been previously shown that intravascular neutrophil adhesion *per se* induces alterations in vascular permeability supporting a role for these cells in BBB breakdown (84–86). The inhibition of MPO and elastase in EAE mice reduced the number of infiltrating neutrophils, restored the integrity of the BBB, and attenuated the clinical symptoms (58, 59). MPO-producing cells were also identified in the brain parenchyma of AD patients and corresponding animal models (10). MPO and elastase are involved in the production of NETs, whose release in the CNS correlates with neuronal damage and BBB breakdown (57, 87). Indeed, the formation of NETs occurs in both MS and AD, strongly suggesting a role for neutrophils in the brain damage associated with both diseases (10, 40). Moreover, circulating neutrophils from MS and AD patients display a stronger oxidative burst, which may contribute to the formation of NETs, the activation of matrix metalloproteinases (MMPs), and therefore to BBB breakdown (40–42) (**Figure 1**, **Table 1**).

Taken together, these data suggest that activated circulating neutrophils mediate BBB damage and neurotoxicity in MS and AD by producing inflammatory mediators such as MPO and ROS, and by releasing NETs (88, 89). The blocking or inhibition of neutrophil activity could therefore achieve therapeutic benefits for both MS and AD patients (**Figure 1**).

THE ROLE OF HETEROGENEOUS MONOCYTES IN MS AND AD

Circulating monocytes are heterogeneous and plastic innate immune cells that can promptly respond to changes in their environment. Traditionally, human monocytes are divided into three subsets: (i) classical ($CD14^+/CD16^-$), (ii) intermediate ($CD14^+/CD16^+$), and (iii) non-classical ($CD14^{lo}/CD16^+$). In mice, only two subclasses of monocytes have been identified: (i) classical ($CCR2^+/CX3CR1^-/Ly6C^{hi}$), and (ii) non-classical ($CCR2^-/CX3CR1^+/Ly6C^{lo}$) (90). In addition to the differential expression of surface markers, these subsets show transcriptional and functional differences: classical monocytes are the main subset recruited to sites of infection and injury (91), whereas non-classical monocytes circulate in the blood, patrolling the vasculature (92).

Systemic alterations in monocyte subsets have been reported in humans with neurodegenerative disorders. The dysregulation of monocyte subsets in patients with RRMS involves the expansion of non-classical and intermediate monocytes and the depletion of classical monocytes (61, 62). Similarly, AD patients with symptoms ranging from very mild to severe dementia accumulate non-classical and intermediate monocytes at the expense of classical monocytes (63) (**Figure 1**, **Table 1**). Although changes in circulating monocyte subsets have been confirmed across diverse CNS diseases, the meaning, causes and consequences of these alterations are still unclear. Whether different monocyte subsets correspond to developmental stages or whether each monocyte subset has a different developmental pathway remains to be determined. Given the plasticity of monocytes and their ability to respond rapidly to a wide variety of stimuli, the analysis of monocyte activation, and cytokine profiles should indicate their function and contribution to peripheral inflammation during neurodegeneration. Indeed, circulating monocytes in MS and AD patients shift toward

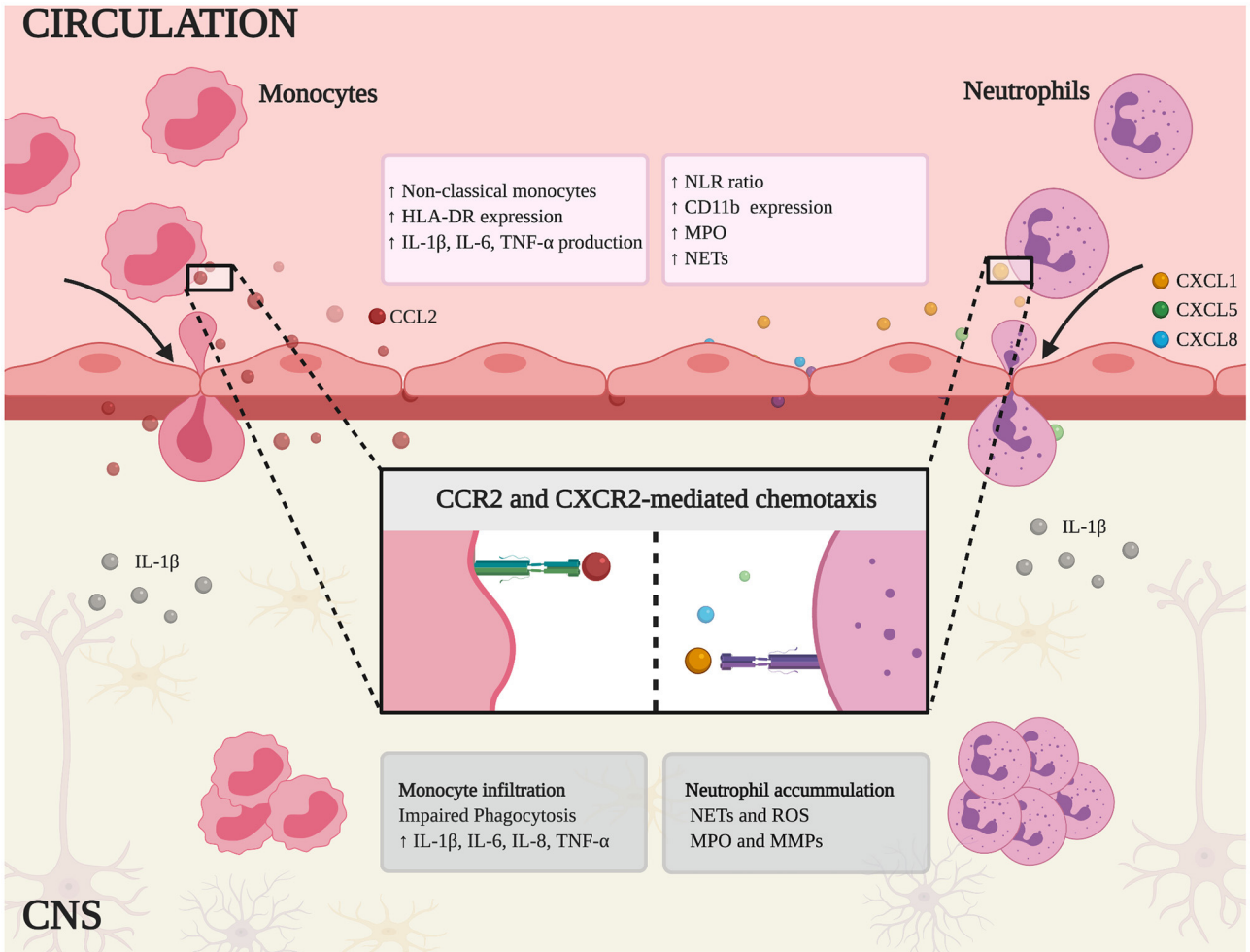

FIGURE 1 | Schematic representation of innate immune mechanisms in MS and AD. Non-classical monocytes and neutrophils are expanded in peripheral blood and the higher neutrophil-lymphocyte ratio (NLR) correlates with the clinical symptoms of each disease. In addition, these cells express canonical activation markers (HLA-DR and CD11b) and release inflammatory mediators, such as pro-inflammatory cytokines (IL-1β, IL-6, TNF-α), myeloperoxidase (MPO), and neutrophil extracellular traps (NETs). Chemokine binding stimulates monocytes and neutrophils to infiltrate the CNS by chemotaxis (mediated by CCR2 and CXCR2, respectively). Within the CNS, monocytes show impaired phagocytosis and secrete the pro-inflammatory cytokines IL-1β, TNF-α, IL-6, and IL-8, thus fueling the inflammatory response. Similarly, infiltrating neutrophils contribute to neuroinflammation and tissue damage by releasing NETs, reactive oxygen species (ROS), MPO, and matrix metal proteinases (MMPs).

a pro-inflammatory phenotype (63, 64) (**Figure 1**, **Table 1**). Particularly, in MS patients, isolated monocytes were shown to produce more IL-1β, TNF-α, IL-6, and IL-8 under basal conditions (61). Similarly, unstimulated monocytes from patients with dementia express higher levels of *IL-6*, *IL-1β*, and *TNF-α* mRNA (63) (**Figure 1**), suggesting a pro-inflammatory phenotype in AD. Interestingly, *IL-8*, and *TNF-α* mRNA levels also increase when human monocyte-like cell line THP-1 is incubated with plasma from AD patients or transgenic mice with AD-like disease, compared to plasma from healthy human controls or wild-type mice, respectively (65). This provides evidence that systemic inflammatory conditions affect the function of circulating monocytes.

Monocytes can migrate into tissues and differentiate into macrophages, making them important players in brain homeostasis. However, the correct identification of these cells in the CNS has proved challenging due to the similarities between microglia and monocyte-derived macrophages, especially during inflammation when the surface markers change. This has been addressed by advances in genome editing and cell tracing technology, leading to the identification of CCR2 and CX3CR1 as markers of murine classical monocytes and microglia, respectively (93). In this context, establishing how circulating monocytes are recruited to the inflamed brain can contribute significantly to the understanding of pathophysiology in MS and AD. Studies in murine models revealed the essential role of CCR2, a chemokine receptor involved in mononuclear trafficking at inflammation sites (94). In the EAE model of MS, classical monocytes were found to infiltrate the inflamed brain and the ablation of CCR2 blocked this process, suggesting a key role for

TABLE 1 | Summary of common pathways in the innate immune system during the development of MS and AD.

Common mechanisms	MS	AD
CNS neutrophil infiltration is related to disease progression	(26) (human) (27) (human) (28) (mouse) (29) (mouse)	(10) (mouse)
High NLR correlates with disease progression	(33) (human) (34) (human)	(32) (human) (35) (human)
Circulating neutrophils display a primed-activated phenotype	(36) (human) (38) (human) (40) (human)	(37) (human) (39) (human) (41) (human) (42) (human) (43) (mouse)
CD11b expression on circulating neutrophils correlates with disease progression	(36) (human)	(37) (human)
CXCL8 is elevated in the plasma and CSF and is related to disease activity	(44) (human) (45) (human)	(46) (human) (47) (human) (48) (human)
Elevated CXCL1 expression in the CNS is related to clinical impairment	(36) (human) (44) (human) (49) (mouse)	(50) (human) (51) (mouse)
Elevated IL-1 expression in the CNS	(52) (human) (53) (mouse)	(54) (human) (55) (mouse)
Increased levels of MPO in the blood and CNS correlates with neuropathology	(56) (human) (57) (human) (58) (mouse) (59) (mouse)	(46) (human) (60) (human) (10) (human and mouse)
Circulating neutrophils show a more intense oxidative burst	(40) (human)	(41) (human) (42) (human)
Systemic phenotype alteration in circulating monocytes (increased frequency of non-classical monocytes at the expense of classical ones)	(61) (human) (62) (human)	(63) (human)
Circulating monocytes display a pro-inflammatory state	(64) (human)	(63) (human) (65) (human and mouse)
CCR2 is involved in monocyte CNS invasion	(66) (mouse)	(67) (mouse) (68) (mouse)
Monocytes display impaired phagocytosis and an enhanced pro-inflammatory phenotype	(69) (human)	(70) (human)

this receptor in the recruitment of monocytes to the CNS during EAE (66). The same phenomenon has been observed in AD mice, where the loss of CCR2 reduces the number of monocytic phagocytes in the brain (67, 68) (**Figure 1**, **Table 2**). Together, these findings suggest that classical monocytes are the main subset that invades the CNS in neuroinflammatory conditions such as MS and AD and that CCR2 plays a fundamental role in the recruitment of these cells into the CNS. Interestingly, while CCR2 blockade in EAE has a beneficial effect, inhibition of this receptor in AD models increases Aβ deposition and worsens memory deficits, suggesting a decreased expression of CCR2 could play a potential role in the etiology of AD. Accordingly, the number of monocytes is lower in AD mouse models than controls, mostly due to the depletion of $CCR2^{+}$ monocytes, suggesting these cells are severely impaired in AD (140). Also, in AD patients, CCR2 expression decreases in circulating monocytes whereas the levels of plasma CCL2 were increased, suggesting systemic immunologic dysfunction CCR2-CCL2 axis (141, 142) (**Figure 1**). Although mouse models of MS confirm the close involvement of monocytes in disease pathogenesis, clinical trials using CCR2 antagonists did not demonstrate efficacy (EU Clinical Trials: https://www.clinicaltrialsregister.eu/ctr-search/search?query=2004-000073-64). Indeed, $CCR2^{+}$ monocytes can be immunosuppressive, they replenish important macrophage populations, and they play pivotal roles during infection, potentially explaining the lack of positive results following a CCR2 therapeutic blockade in MS (68, 143, 144).

Infiltrating monocytes and resident microglial cells can both react to inflammatory stimuli and mount an immune response in the CNS during neurodegenerative diseases (144). Indeed, monocytes not only infiltrate the CNS parenchyma but also colonize the meninges in EAE mice (145). Furthermore, monocyte infiltration begins at the onset of the disease and continues to increase until the disease peak, suggesting a role for these cells in disease induction and progression (145). Moreover, monocyte-derived cells infiltrating the CNS are major players in antigen presentation during EAE and recent studies identified several subtypes of infiltrating monocytes/myeloid cells in the CNS with different transcriptional landscapes during the acute and chronic stages of EAE (146). Similarly, single-cell studies revealed disease-specific transformations across several types of brain-associated phagocytes in murine models of AD, but the existence of common signatures between EAE/MS and AD is unclear (147). In AD mice, circulating monocytes have been shown to invade the brain and reduce amyloid β burden, suggesting a beneficial role for these cells in AD (148). Also, patrolling monocytes have been described to crawl onto the luminal walls of amyloid β-positive veins, suggesting their ability to target and clear amyloid β (149). The same applies to perivascular macrophages in another mouse model of AD, in which their depletion led to an increased accumulation of amyloid β deposits in blood vessels (150). Brain macrophages are not only involved in the clearance of CNS debris or amyloid β, but also play an important role in regulating iron levels. Extracellular accumulation of iron during neurodegeneration can be attributed to an array of processes including oligodendrocyte and myelin degeneration (151, 152). Indeed, increased iron deposits in white matter lesions have been shown in both MS and AD, and iron accumulation correlates with cognitive deficits (153, 154). Interestingly, the deposition of iron observed in MS was often co-localized with microglia/macrophages, which express the transferrin receptor, a main iron influx protein. By capturing iron and, therefore, preventing Fenton reactions and the creation of oxygen radicals, macrophages play an important regulatory function in the inflamed brain during MS (155, 156). In AD, however, there is limited information on iron uptake by macrophages, although recent evidence suggests that stimulation of microglia with Aβ increases the uptake of non-bound iron by these cells (157).

However, although monocytes infiltrate the brain and, to some degree, remove debris, iron and amyloid β, these cells in mice with AD-like disease are ineffective in clearing amyloid β in the diseased brain and their peripheral phenotype changes

TABLE 2 | Summary of common pathways in the adaptive immune system during the development of MS and AD.

Common mechanisms	MS	AD
$CD4^+$ cells infiltrate CNS	(95) (human) (96) (human) (97) (human)	(98) (human) (99) (human) (100) (human)
Increased frequency of circulating Th17 cells and serum level of IL-17	(101) (human) (102) (human)	(103) (human) (104) (human)
α4-integrin is involved in CNS invasion by $CD4^+$ T cells	(105) (mouse) (106) (human)	(31) (mouse) (107) (mouse)
$CD4^+$ T cells interact with microglia expressing MHC-II at high levels	(108) (human) (109) (rat)	(110) (human) (111) (mouse)
$CD4^+$ T cells work along the gut–brain axis to modify cognitive functions	(112) (mouse)	(113) (mouse) (114) (mouse and human)
$CD8^+$ T cells infiltrate the CNS and trigger detrimental effects	(115) (human) (116) (human)	(100) (human) (117) (human) (98) (human) (118) (mouse)
Increased proportion of circulating $CD8^+$ T cells	(119) (human) (120) (human)	(121) (human)
Circulating $CD8^+$ T cells show a primed-activated phenotype	(122) (human) (123) (human) (124) (human)	(117) (human) (125) (human)
Clonally expanded CD 8^+ T_{EMRA} cells	(126) (human)	(117) (human)
CD 8^+ T cells clonally respond against EBV	(127) (human)	(117) (human)
Lower number of circulating $CD4^+$ $CD25^+$ $FoxP3^+$ cells	(128) (human) (129) (human)	(130) (human)
T_{reg} cells with impaired regulatory activity	(131) (human) (132) (human)	(133) (human) (134) (human)
Depletion of T_{reg} cells associated with worst outcomes	(135) (mouse) (136) (mouse) (137) (mouse)	(138) (mouse) (139) (mouse)

to a pro-inflammatory profile with limited phagocytic ability. Indeed, peripheral blood monocytes from AD patients cannot differentiate normally *in vitro* and have a lower capacity for the uptake of amyloid β uptake, eventually leading to apoptosis (70). Similarly, circulating monocytes in MS patients also adopt a pro-inflammatory profile with limited phagocytic ability, thus failing to promote remyelination and repair through the removal of myelin debris by phagocytosis (69) (**Figure 1**, **Table 1**). Collectively, these data suggest that chronic systemic inflammation in MS and AD leads to common pathological changes among the population of circulating monocytes. Understanding the role of these cells may provide insight into the disease mechanisms and lead to new therapeutic targets in neurodegenerative disorders.

THE ROLE OF T CELLS IN THE DEVELOPMENT OF MS AND AD

T lymphocytes are cells of the adaptive immune system that provide specific responses to eradicate pathogens or antigens that act as elicitors (158). Depending on their function, T lymphocytes can be subdivided into three main classes: (i) $CD8^+$ cytotoxic T (Tc) lymphocytes responsible for the elimination of infected somatic cells, (ii) $CD4^+$ T helper (Th) lymphocytes that assist and guide other immune cells, and (iii) regulatory T (T_{reg}) lymphocytes associated with the attenuation and resolution of inflammation. The pathological dysregulation of the adaptive immune system promotes chronic and uncontrolled inflammatory reactions that may eventually lead to tissue damage. MS and AD are both characterized by a chronic neuroinflammatory pathology (159, 160). In MS, T cells are known to play an essential role in disease pathogenesis but the extent to which T cells contribute to the pathology of AD is less clear. In MS, T cells react against myelin autoantigens, migrate across a damaged BBB, accumulate in active lesions, and trigger damage to myelin and underlying axons, thus promoting all classical MS symptoms (161). Similarly, the post-mortem analysis of brains from AD patients revealed the accumulation of brain-infiltrating T cells, and recent experimental evidence from animal models of AD suggest a pathological role for these cells (100, 117). In support of this, the sequestration of T lymphocytes in lymphoid organs induced by fingolimod treatment decreases the number of circulating T cells and ameliorates disease in MS patients and animal models of AD (162–166).

$CD4^+$ T Lymphocytes in MS and AD

$CD4^+$ T cells infiltrate both the white and gray matter of MS patients, and similar observations have been reported in AD brains (95–100) (**Table 2**). Pathogenic $CD4^+$ T cells in MS and EAE have been subtyped by cytokine profiling, revealing Th1 cells that produce IFN-γ and Th17 cells that produce IL-17. Both Th1 and Th17 cells are key components of the autoimmune inflammatory process during the development of MS, and they may fulfill analogous roles in AD. Following CNS invasion, Th1 and Th17 cells produce inflammatory mediators and cytokines to establish and/or maintain an inflammatory environment that promotes neuronal loss, a common feature of MS and AD that positively correlates with the disease course (167, 168). In particular, the infiltration of Th17 cells into the CNS of MS patients increases the concentration of IL-17 in the blood and CSF and the number of Th17 cells found in these compartments (101, 102, 169). Similarly, IL-17 also accumulates to higher levels in the serum of AD patients compared to healthy controls and has been proposed as part of a blood-based signature to distinguish individuals with AD from healthy subjects (104, 170). The population of circulating Th17 cells has also been shown to increase in MCI patients compared to both age-matched controls and AD patients, suggesting that Th17 cells may be involved in the early stages of AD (103) (**Table 2**). Intriguingly, EAE mice modified to abolish IL-17 production, as well as AD models treated with neutralizing antibodies against IL-17, show delayed clinical progression, confirming the harmful effects of IL-17 in EAE and suggesting that Th17 cells also contribute to the progression of AD (171).

$CD4^+$ T cells appear to invade the CNS of AD and MS patients using common molecular pathways, emphasizing the common leukocyte recruitment mechanisms in these two diseases. For example, several studies in MS/EAE have shown that α4-integrins play a key role in the trafficking of Th cells (105, 106). EAE

progression is delayed following the selective deletion of α4-integrin genes in T cells and MS progression is delayed by treatment with the α4-integrin-blocking humanized antibody natalizumab (172, 173). We and others recently demonstrated a similar molecular mechanism controlling the infiltration of Th cells in AD models (31, 107). We observed the stronger expression of α4-integrins on circulating $CD4^+$ T cells in an AD mouse model compared to age-matched controls, along with an increase in the abundance of $CD4^+$ cells in the brains of AD mice (31) (**Figure 2**, **Table 2**). Importantly, blocking α4-integrins inhibited the adhesion of circulating leukocytes in the brain microcirculation and reduced the neuropathological hallmarks of AD, highlighting the potential for a therapeutic approach that is similar in efficacy to the use of natalizumab in MS patients (172, 173).

In EAE models, lymphocytes are presented with antigens in the periphery before CNS invasion. Indeed, T-cell priming begins in secondary lymphoid organs and leads to the activation and expansion of neuroantigen-reactive T cells that later infiltrate the CNS, where they re-encounter their cognate antigen (174). Within the CNS, microglial cells may promote the proliferation and activation of CNS-reactive T lymphocytes (174, 175). In both MS and AD, activated microglia express the main major histocompatibility complex class II molecule (MHC-II) as well as co-stimulatory molecules such as CD40, CD80, and CD86, which equip the microglia for antigen presentation to infiltrating T cells, creating a vicious cycle that promotes neuroinflammation and potentially antigen presentation (108–111) (**Figure 2**, **Table 2**). As a result, the pro-inflammatory environment that can activate $CD4^+$ T cells is continuously boosted, and may promote neuronal damage in both MS and AD (**Figure 2**).

Although $CD4^+$ T cells are considered pathogenic in several CNS disorders, they may also provide beneficial functions in AD, ranging from tissue protection to regeneration (176). In AD models, amyloid β-reactive T cells effectively target amyloid β plaques in the brain, enhancing phagocytosis by microglia and leading to neuronal repair (177). Furthermore, Th1 cells injected into the ventricles of AD mice were able to induce the differentiation of microglia (protective MHC-II^+ subtype), boosting the capacity for amyloid β clearance (111). Despite these tantalizing results, a protective role for $CD4^+$ T cells has yet to be confirmed in AD, and immunotherapeutic approaches based on amyloid β have not achieved efficacy in clinical trials (178).

T_{reg} Cells and Their Failure to Control Inflammation in MS and AD

T_{reg} cells fulfill an active regulatory role in peripheral tolerance mechanisms, preventing the onset of autoimmunity and limiting chronic inflammation. They downregulate the activities of various immune cells, including effector T cell functions and proliferation, by the secretion of immunosuppressive cytokines (including TGF-β, IL-10 and IL-35) and/or by direct cytotoxicity and the induction of apoptosis (through the release of granzyme B and perforin 1) (179–181). T_{reg} cells also cause indirect immunosuppression via cytotoxic T lymphocyte antigen 4 (CTLA4), CD39, and CD73, and disrupt the metabolism of the effector T cells by modulating the maturation and/or function of the dendritic cells (DCs) required for their activation (182).

Dysfunctional T_{reg} cells have been linked to neuroinflammatory conditions, and the analysis of peripheral blood demonstrates how T_{reg} cells can contribute during neurodegenerative diseases. T_{reg} cells have recently been shown to infiltrate the brain and suppress astrogliosis by producing amphiregulin in a model of ischemic stroke, but their role during AD and MS is unclear (183). Several studies have shown that the number of circulating T_{reg} cells declines in both MS and AD patients compared to matched controls, suggesting that their dysregulation in the periphery reduces their immunosuppressive capacity and promotes uncontrolled inflammation (128–130). Indeed, T_{reg} cells isolated from the peripheral blood of MS patients show an impaired ability to modulate $CD4^+$ T cell proliferation and IFN-γ production (131). Compared to healthy controls, RRMS patients also produce more Th1-like $Foxp3^+$ T cells that secrete IFN-γ and show a limited reduced immunosuppressive capacity (132). Similarly, the immunosuppressive functions of T_{reg} cells in AD patients are compromised compared to both healthy controls and subjects with MCI (133, 134) (**Table 2**). These data are supported by animal models discussed below, where T_{reg} cells appear to be important in both EAE and AD, especially during the early phases of both diseases, but at later stages they are depleted and/or dysfunctional and are therefore unable to control the inflammatory response.

The depletion of T_{reg} cells by anti-CD25 antibodies in EAE mice increased the severity of the disease, boosting the production of IL-17 and T cell infiltration (135). In line with the protective role of T_{reg} cells, the transplantation of neural stem cells in EAE mice induces remyelination by expanding the T_{reg} cell population (136, 137). Similarly, the depletion of T_{reg} cells in mice with AD-like disease is associated with the premature loss of cognitive functions and a worse outcome (138). Furthermore, low doses of IL-2 increased the number of T_{reg} cells in the peripheral blood and lymphoid organs of these mice, restoring their cognitive ability (138). Similar results were reported by others, showing that T_{reg} cell depletion for 4 months during the early stages of AD-like disease aggravated the cognitive deficits and increased the deposition of amyloid β plaques (139). In these studies, the adoptive transfer of purified T_{reg} cells improved cognitive functions and reduced the amyloid β burden (139) (**Table 2**). Curiously, T_{reg} cell depletion during the late disease stage in an aggressive model of amyloidosis also conferred a beneficial effect, presumably by boosting the immune response, suggesting that the effect of T_{reg} cells on AD-like disease is stage-dependent (184). Taken together, these results suggest that T_{reg} cells play a key role in controlling the development of neuroinflammation in both MS and AD (**Figure 2**). Therapeutic strategies aiming to exploit the immunosuppressive properties of T_{reg} cells may therefore help to address both pathologies.

$CD8^+$ T Lymphocytes in AD and MS

$CD8^+$ T cells appear less heterogeneous than $CD4^+$ T cells, but the functional classification of this population is not completely

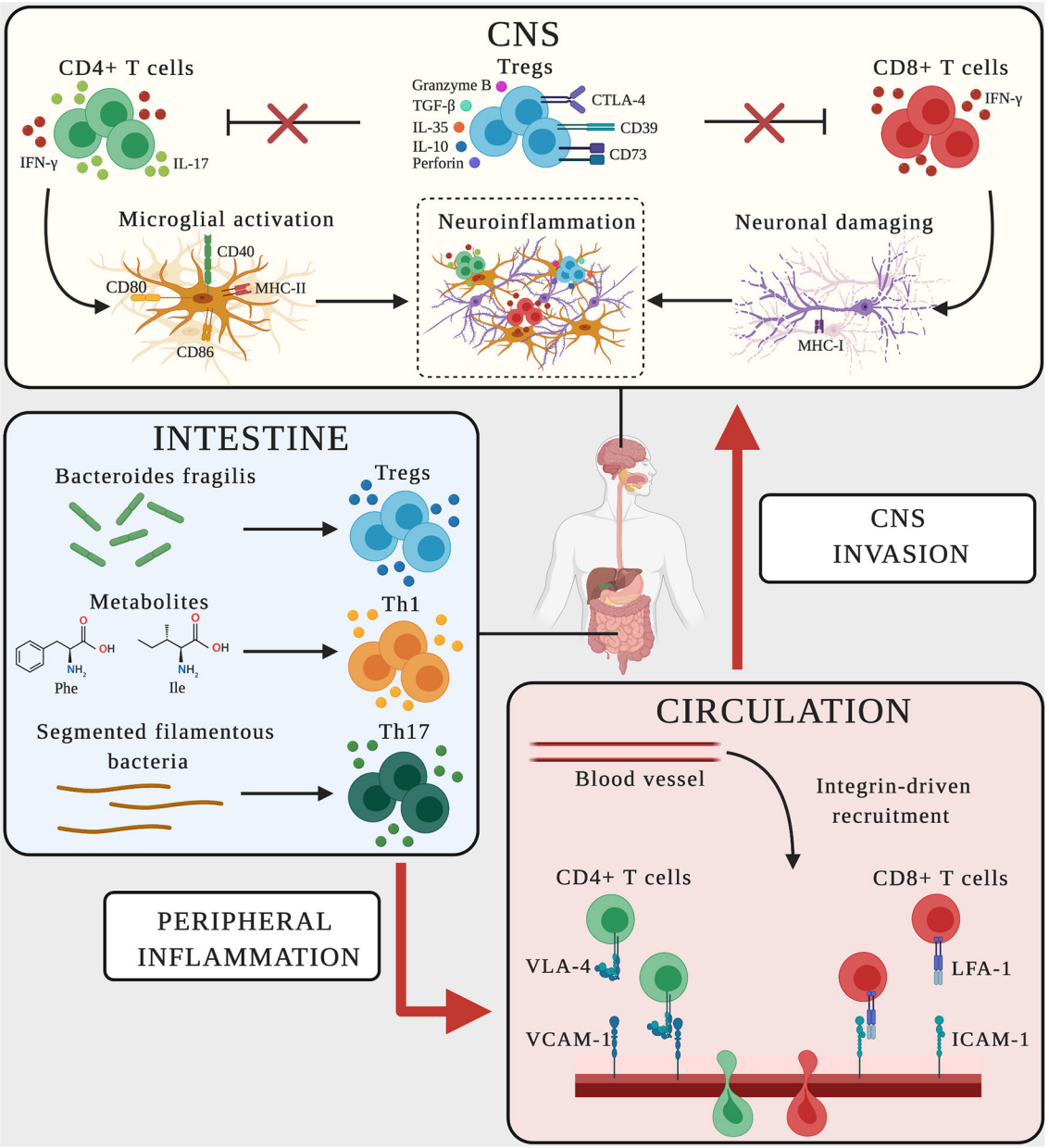

FIGURE 2 | Schematic representation of adaptive immune mechanisms in MS and AD. In CNS, $CD4^+$ T cells producing the pro-inflammatory mediators IFN-γ-and IL-17 promote microglial activation, upregulating the expression of CD40, MHC-II, CD80, and CD86 on the surface and favoring neuroinflammation. $CD8^+$ T cells producing IFN-γ bind neurons expressing MHC-I, triggering neuronal damage and boosting neuroinflammation. Dysregulated T_{reg} cells fail to suppress effector T cell functions (red crosses), thus sustaining the neuroinflammatory environment. In the intestine, the microbiome and its metabolites influence the polarization and activation of T cells. *Bacteroides fragilis* promotes the expansion of T_{reg} cells, the amino acids phenylalanine and isoleucine induce the differentiation of Th1 cells, and segmented filamentous bacteria trigger Th17 cell polarization. Vascular endothelial cells express the LFA-1 and VLA-4 counter-ligands (ICAM-1 and VCAM-1) guiding the transmigration of peripherally activated T cells from the circulation to the CNS.

clear. Nevertheless, cytotoxic lymphocytes play a prominent role in the development of many viral and non-viral diseases by the direct killing of infected or otherwise modified cells. Following antigen recognition, cytotoxic T cells classically induce apoptosis in target cells via two alternative mechanisms: (i) FasL-CD95 (FasR) binding to activate caspase, and (ii) the release of granzyme B and perforin. $CD8^+$ effector T cells may also cause cellular damage indirectly by secreting the pro-inflammatory cytokines TNF-α and IFN-γ (185). During immune responses, $CD8^+$ T cells interact with $CD4^+$ T cells to optimize the precision

of $CD8^+$ T cell effector functions after priming by enhancing their cytotoxicity and capacity for migration (186).

$CD8^+$ T cells have received less attention than $CD4^+$ T cells in MS and EAE, but it is now well-established that $CD8^+$ T cells are more activated in the periphery and can infiltrate active lesions, contributing to the increasing severity of MS symptoms (115, 116, 187). Furthermore, clonally expanded cytotoxic T cells exacerbate brain inflammation in EAE initiated by $CD4^+$ T cells, suggesting that $CD8^+$ T cells may be primarily responsible for the observed cerebral alterations (187) (**Table 2**).

The role of $CD8^+$ T cells in AD is less clear, although these cells were first detected in the brains of AD patients almost 20 years ago (100). The infiltration of $CD8^+$ T cells into the brains of AD patients and corresponding mouse models also correlates with disease worsening, suggesting these cells may be involved in disease development (98, 100, 118). $CD8^+$ T cells were shown to accumulate in an active state in the peripheral blood of MS patients and in AD patients with dementia (119–121) (**Table 2**). The comparison of blood samples from AD patients and healthy controls revealed the production of more pro-inflammatory cytokines by cytotoxic T cells in the AD patients and a greater proportion of activated HLA-DR^+ $CD8^+$ T cells (117, 125). Similarly, circulating $CD8^+$ T cells from patients in the acute phase of RRMS showed increased adhesion to brain venules compared to control cells, further highlighting their activated phenotype (122). In line with this, the characterization of $CD8^+$ T cells from the CSF and brain tissue of MS patients showed their activated/memory phenotype (123, 124) (**Table 2**). Notably, a higher frequency of $CD8^+$ effector memory T cells was detected in the CSF of AD patients with dementia compared to controls, suggesting $CD8^+$ T cells contribute to brain damage in both AD and MS with similar underlying mechanisms (117, 121).

The extravasation of cytotoxic T cells in the brain promotes the brain damage caused by $CD8^+$ T cells and appears to be mediated by LFA-1 integrin (CD11a/CD18) in both MS and AD (**Figure 2**). LFA-1 is a marker of leukocyte activation that binds ICAM-1, which is overexpressed by endothelial cells in several neuroinflammatory conditions (10, 188). CD11a expression was shown to increase on clonally expanded $CD8^+$ T cells in MS patients, promoting their transmigration into the brain (116). Without referring to specific T cell populations, previous studies have shown that the transfer of encephalitogenic $CD11a^{-/-}$ T cells to wild-type mice reduces the severity of EAE, suggesting that LFA-1 also facilitates the migration of $CD8^+$ T cells in this disease (189). The presence of LFA-1^+ T cells infiltrating the hippocampus of AD patients suggests a role for LFA-1 also in AD (100). This is consistent with the increase in *Itgb2* (LFA-1) mRNA levels in the hippocampus of a mouse model of tauopathy (190). Finally, the strong upregulation of ICAM-1 was observed in cortical and hippocampal brain regions invaded by $CD3^+$ T cells in mouse models of AD, suggesting that LFA-1 also promotes T cell migration into the AD brain (10, 191). Further studies are needed to confirm that LFA-1 is required for the trafficking of $CD8^+$ T cells in AD, but given that LFA-1 is required for cytotoxic T cell activation, maturation, immuno-synapse stabilization and functioning, this integrin is likely to play a key role in driving $CD8^+$ responses in AD (192).

Another important topic in the context of MS and AD is the antigenic specificity and clonal origin of CNS-infiltrating $CD8^+$ T cells. The analysis of lymphocytes collected from the blood, CSF and brain lesions of many MS patients has shown that $CD8^+$ T cells undergo clonal expansion, suggesting that they are activated by specific antigens (115, 123). Other studies in MS patients have identified a population of $CD8^+$ T cells specific for myelin proteins, presenting an activated/memory phenotype due to *in situ* antigen presentation (96, 119, 120, 193). Tau protein may fulfill a similar role in AD, representing the potential link between cytotoxic T cells and disease development. Indeed, $CD8^+$ T cells accumulate in the hippocampal regions of a mouse model of tau pathology, apparently via a tau-driven transmigration mechanism (190). A correlation between tau pathology and the infiltration of $CD3^+$ T cells was revealed by the immunohistochemical analysis of post-mortem AD brains, further supporting the potential association between tau and $CD8^+$ T cell-dependent pathology in AD (194).

Clonally expanded $CD8^+$ T_{EMRA} cells were recently identified in the CSF of AD patients, suggesting that antigen-experienced cytotoxic cells patrol the intrathecal space of AD patients. Interestingly, as already shown in MS patients, $CD8^+$ T cells in the CSF were expanded predominantly against Epstein-Barr viral antigens, suggesting a new link between EBV, $CD8^+$ T cells and AD (117, 127). $CD8^+$ T_{EMRA} cells accumulated not only in the CSF of AD patients, but also in the blood of RRMS and SPMS patients, suggesting these cells contribute to the progression of both AD and MS by promoting chronic inflammation (126) (**Table 2**). More detailed studies are required to determine precisely how this cell population fits into the complex pathogenesis of neurodegenerative disorders.

The pro-inflammatory nature of $CD8^+$ T_{EMRA} cells is strictly associated with IFN-γ secretion, which occurs mainly during active proliferation (195). One of the harmful functions of IFN-γ is the ability to favor neuronal killing by $CD8^+$ T cells via the FasR-FasL pathway (185). Accordingly, previous studies have shown that the overexpression of MHC-I on neurons exposed to IFN-γ promotes neuronal damage by $CD8^+$ T cells via TCR-MHC-I binding (196). Interestingly, MHC-I has been detected on neurons in both hippocampal and cortical brain regions, which are heavily damaged in AD (196). Similarly, $CD8^+$ T cells in chronic and active MS plaques were found marginally in contact with oligodendrocytes, astrocytes and neurons expressing MHC-I at high levels (96, 96, 193). These results indicate that $CD8^+$ T cells producing IFN-γ enhance the expression of MHC-I on neurons in MS and AD, and could therefore promote the brain alterations associated with the progression of both diseases (**Figure 2**). In conclusion, $CD8^+$ T cells appear to drive the development of both MS and AD by sustaining chronic inflammation and directly causing CNS injury.

The Gut–Lymphocyte–Brain Axis in AD and MS

Several clinical and preclinical studies have shown that the course of MS and AD is influenced by the commensal gut

microbiome, highlighting the interplay between the brain, gut microbes, intestinal barrier and immune system (197, 198). Indeed, fecal and mucosa-associated gastrointestinal tract microbes differ between AD patients and healthy controls, and recent studies comparing germ-free animals and those exposed to pathogenic bacteria, probiotics or antibiotics suggested a role for gut microbes in host cognitive functions and the development of AD-like neuropathological features (113, 199–202). Many studies have also focused on the role of gut microbes in MS and EAE (112, 203–208). These studies suggest that altering the gut microbiome with antibiotic cocktails or probiotics can attenuate the disease course by modulating regulatory immune responses (198). Although many of the microbes and metabolites in the gut–CNS axis have been identified, little is known about the underlying cellular and molecular immune mechanisms. In MS, the balance between pro-inflammatory myelin-reactive effector cells and anti-inflammatory immune elements controlling the formation of CNS lesions is continuously influenced by the gut. Indeed, both monocytes and gut-resident macrophages influence the gut-dependent activation of $CD4^+$ T cells, which promotes Th1 polarization and IFN-γ secretion (209). Gut microbes and their metabolites also regulate T cell-mediated adaptive immune responses. For example, specific bacteria such as *Akkermansia muciniphila* and *Acinetobacter calcoaceticus* are associated with MS, inducing a pro-inflammatory T cell phenotype that perpetuates autoimmune responses, whereas segmented filamentous bacteria, symbiotic colonizers of the small intestine, induce Th17 differentiation and activation to promote the development of EAE (210, 211) (**Figure 2**). In contrast, CNS demyelination and inflammation during EAE is inhibited by gut flora rich in the commensal bacterium *Bacteroides fragilis*, which promotes the expansion of T_{reg} cells expressing the ectonucleotidase CD39 and their migration into the CNS (212) (**Figure 2**). A recent innovative study in SPMS patients confirmed a role for the gut–brain axis in MS patients, showing the depletion of a subset of circulating memory $CD4^+$ T cells expressing the gut-homing chemokine receptor CCR9 and the α4β7 adhesion molecule and a tendency to switch from a regulatory to a pro-inflammatory phenotype that produces more IFN-γ and IL-17 (213). Recently, gut dysbiosis in MCI patients was shown to increase phenylalanine and isoleucine levels, correlating with an increase in the number of circulating Th1 cells (114) (**Figure 2**). Interestingly, naive $CD4^+$ T cells exposed to phenylalanine or isoleucine acquire an activated Th1 phenotype, and the oral treatment of mouse models of AD with the prebiotic oligosaccharide GV-971 (which suppresses gut dysbiosis) reduced the concentration of phenylalanine and isoleucine, resulting in the amelioration of neuroinflammation and cognitive impairment (114).

Collectively, these data show that gut dysbiosis contributes to peripheral immune cell dysregulation and triggers an enhanced inflammatory immune response. The gut–brain axis may therefore provide an appropriate target for immunomodulatory therapy in both diseases.

CONCLUSIONS

Neurodegenerative diseases are increasing in prevalence and socioeconomic impact. The identification of common cellular and molecular mechanisms involving the immune system may provide more insight into pathogenesis, leading to potential common therapeutic strategies. Several genome-wide association studies (GWAS) in both MS and AD patients have revealed associations between these diseases and gene expression in peripheral adaptive and innate immune cells, suggesting common immune mechanisms controlling neuroinflammation and neurodegeneration (214–216). Moreover, functional studies *in vitro* and *in vivo* have revealed shared detrimental molecular mechanisms in peripheral leukocytes, representing potential common therapeutic targets for the control of immune responses in MS and AD (217–219). One clear example of such a shared molecular mechanism is represented by α4 integrins, which can be targeted in both MS and AD. Indeed, the therapeutic effect of α4 integrins in EAE and MS has been corroborated by recent studies showing that targeting these adhesion molecules reduces neuroinflammation and the neuropathological hallmarks of AD (31). Moreover, reducing BBB breakdown that characterizes several neurodegenerative disorders, including MS and AD, could be considered as another common possible therapeutic strategy in reducing not only the influx of various neurotoxic agents, but also the recruitment of immune cells into the CNS (220, 221). By using animal models of both MS and AD, it was indeed demonstrated that targeting BBB pathways to preserve vascular integrity, ameliorates the course of brain pathology (222, 223). Therefore, a deeper understanding of the activation status of peripheral innate and adaptive immune cells in the blood, their trafficking mechanisms, CNS pathogenic signatures, and neurotoxic effects, may lead to the discovery of new common biomarkers of MS and AD that could facilitate the identification of common therapeutic strategies for both diseases.

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All authors contributed to the literature search and writing the review.

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Alpha-Synuclein in the Regulation of Brain Endothelial and Perivascular Cells: Gaps and Future Perspectives

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Misfolded proteins, inflammation, and vascular alterations are common pathological hallmarks of neurodegenerative diseases. Alpha-synuclein is a small synaptic protein that was identified as a major component of Lewy bodies and Lewy neurites in the brain of patients affected by Parkinson's disease (PD), Lewy body dementia (LBD), and other synucleinopathies. It is mainly involved in the regulation of synaptic vesicle trafficking but can also control mitochondrial/endoplasmic reticulum (ER) homeostasis, lysosome/phagosome function, and cytoskeleton organization. Recent evidence supports that the pathological forms of α-synuclein can also reduce the release of vasoactive and inflammatory mediators from endothelial cells (ECs) and modulates the expression of tight junction (TJ) proteins important for maintaining the blood–brain barrier (BBB). This hints that α-synuclein deposition can affect BBB integrity. Border associated macrophages (BAMs) are brain resident macrophages found in association with the vasculature (PVMs), meninges (MAMs), and choroid plexus (CPMs). Recent findings indicate that these cells play distinct roles in stroke and neurodegenerative disorders. Although many studies have addressed how α-synuclein may modulate microglia, its effect on BAMs has been scarcely investigated. This review aims at summarizing the main findings supporting how α-synuclein can affect ECs and/or BAMs function as well as their interplay and effect on other cells in the brain perivascular environment in physiological and pathological conditions. Gaps of knowledge and new perspectives on how this protein can contribute to neurodegeneration by inducing BBB homeostatic changes in different neurological conditions are highlighted.

Keywords: Parkinson's disease, α-synuclein, endothelial cells, blood brain barrier, border-associated macrophages, perivascular cells

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INTRODUCTION

Neurodegenerative diseases represent a relevant health burden, especially considering the growing population of elderly subjects. Cerebrovascular disorders such as stroke are considered among the major predisposing factors for the development of neurodegenerative diseases, including Alzheimer's disease (AD) and Parkinson's disease (PD) (1, 2). In particular, PD is the second most common neurodegenerative disorder, affecting 2–3% of the population over the age of 65 years

(3). The lack of knowledge on the molecular underpinnings of PD still limits the development of efficient therapies.

Protein aggregates enriched in insoluble α-synuclein fibrils and loss of dopaminergic neurons in the nigrostriatal system are key pathological features of this disorder (4, 5). Of note, the pathological deposition of insoluble α-synuclein at synapses is believed to act as the *primum movens* for neuronal degeneration in PD, as by hindering neurotransmitter release, it can trigger synaptic failure (6–8). This event can then negatively impinge on axonal projections, thus slowly flowing in a retrograde neurodegenerative process culminating in neuronal cell death (6–8). Additionally, α-synuclein-related neuroinflammation, microglia activation, and vascular degeneration (9–12) have been described as important players in disease pathogenesis. This notwithstanding, whether α-synuclein communicates with other neurovascular components such as border-associated macrophages (BAMs) and vascular endothelial cells (ECs), which are involved in the early phases of ischemic brain damage (13–16), remains to be explored.

Alpha-synuclein is a 14 kDa protein owning an undefined structure in aqueous solutions (17). In neurons, the protein regulates various processes including synaptic function, mitochondrial homeostasis, autophagy/lysosomal functions, and cytoskeletal reorganization (8, 18–24). The diverse domains of α-synuclein and its conformational plasticity allow the interaction with a plethora of other proteins and lipid membranes (20). Alpha-synuclein can also undergo post-translational modifications as amino-terminal and carboxy-terminal nitration and phosphorylation [e.g., Ser129 phosphorylation; (25–27)], which in turn can impact its conformation and can lead to the formation of toxic oligomers and fibrils (20). While oligomers can affect membrane permeability as well as neuronal excitability and engulf protein degradation systems (28–30), fibrils can disrupt the integrity of intracellular organelles and induce chronic inflammation (28, 31). In the brain, α-synuclein is expressed not only in the neuronal cells, but at lower levels also in astrocytes, macrophages, and the microglia (32, 33). In the periphery, the protein is expressed in red blood cells (34, 35), platelets (36), and in other immune cells, such as T cells, B cells, natural killer (NK) cells, and monocytes (32). It has been found that α-synuclein can bind microglia cell surface receptors, thus activating intracellular pathways mediating the release of cytokines and upregulating of proinflammatory genes (10, 37). The protein can also regulate ECs function by blocking the exocytosis of Weibel-Palade bodies (WPBs) (38) and by downregulating the expression of tight junction (TJ) proteins (39).

The deposition of α-synuclein insoluble aggregates named Lewy bodies (LB) or glial cytoplasmic inclusions (GCI) characterizes the brain of patients affected by PD and dementia with LB (DLB) or multiple system atrophy (MSA), respectively (5, 40). For this reason, these disorders are commonly referred to as synucleinopathies. Certain pathological strains of α-synuclein, by moving between the brain cells and across the blood–brain barrier (BBB) interfaces and acting as imprinting templates for the pathological conformational shift of other α-synuclein molecules, are believed to mediate the propagation of pathological aggregates within the brain, from the periphery to the brain, or from the brain to the periphery, with a prion-like fashion (41, 42).

This review focuses on how α-synuclein impacts vascular ECs and BAMs regulation and crosstalk. Current gaps and future perspectives in the context of neurological disorders are also presented.

ALPHA-SYNUCLEIN FUNCTIONS IN THE CENTRAL NERVOUS SYSTEM (CNS)

To date, the physiological function of α-synuclein has not been fully disclosed, but we know that it controls neurotransmitter release and synaptic plasticity, particularly inhibiting dopamine overflow and modulating synaptic vesicles storage (20, 43, 44).

The full-length α-synuclein isoform consists of 140 amino acids and its structure can be divided into three main regions. The N-terminal part is essential for membrane binding (45–47) and includes the sites of main familial PD mutations, A30P, A53T, and E46K (18, 20), as well as for several post-translational modifications (48). The central domain, called non-amyloid component (NAC), is hydrophobic and highly aggregation-prone (49), and is necessary and sufficient for α-synuclein fibrillation (50). Finally, the C-terminal region is enriched in negative charges (51) and can interact with the N-terminal domain to form a compact aggregation-resistant structure (52).

Alpha-synuclein is described as an intrinsically disordered protein as it can be found in monomeric form (53) or in a stable tetramer (54) when purified at neutral pH. Rapid environmental changes can induce the formation of partially folded intermediates or kinetically trapped transition states (55). Along aging, the high plasticity of α-synuclein, coupled with post-translational modifications and protein enrichment at synaptic sites, can promote in concert the formation of high molecular weight soluble or insoluble aggregates, such as oligomers, protofibrils, or fibrils (20, 21). In PD, α-synuclein deposition is thought to play a pathogenic role in triggering both central and peripheral neurons degeneration, thus underlying the onset of motor and non-motor symptoms, respectively (56, 57). Interestingly, both monomeric and aggregated α-synuclein can be transferred from cell-to-cell (neuron-neuron, neuron-glia), and also across the BBB, thus contributing to neuropathology spreading (58). Endocytosis, carrier-mediated transports, and tunneling nanotubes are described as the main mechanisms for these exchanges (59, 60). In addition, impairment in glymphatic transport and lymphatic drainage pathways results in the accumulation of α-synuclein in the brain parenchyma and the progression of PD-like pathology in transgenic mouse models (61). This is in line with evidence supporting that general systemic circulation would act as a route for long-distance transmission of endogenous α-synuclein (62).

Alpha-synuclein can also exert a physiological regulatory action on intracellular organelles, including mitochondria (19), endoplasmic reticulum (ER), mitochondria-ER associated membranes (63), Golgi apparatus (64), and nuclei (65). Although the nuclear localization of α-synuclein was the first to be reported,

its involvement in DNA repair mechanisms has been described quite recently (66). Recent findings, showing reduced nucleus to cytoplasmic transport in induced pluripotent stem cell (iPSC)-derived neurons from familial patients with PD bearing A53T mutation or multiplication of the α-synuclein gene locus SNCA (67), support that the protein may also play a role in maintaining nuclear membrane functions. Interestingly, reduced α-synuclein DNA binding associates with transcription deregulation through inhibition of cell cycle-related genes and the nuclear localization of α-synuclein is modulated by its phosphorylation at Serine 129 (68).

The interplay between mitochondria and α-synuclein during the progression of PD still constitutes an issue to be solved, as the exact contribution of mitochondrial deficits and α-synuclein aggregation to dopaminergic neurons degeneration has yet to be clearly elucidated (69, 70). Indeed, the aggregation of α-synuclein induces neural deficits, but it is also evident that mitochondrial dysfunctions are crucial events in the pathogenesis of PD (71, 72). Notably, the observation that α-synuclein is increased following a stroke, and that its induction is involved in the response to post-stroke brain damage, reinforces the idea that the protein can act as a pivotal regulator of neuronal resilience to injury (35, 73–75).

Numerous studies have shown that α-synuclein accumulation and aggregation can activate neuroinflammation (76–79), in agreement with the evidence showing increased levels of tumor necrosis factor alpha (TNFα), interleukin-1β (IL-1β), and IL-6 in the brains of patients with PD (80–82). In particular, reactive microglia have been found in PD brains (83, 84) and in transgenic mouse models of PD, and can be activated by α-synuclein pathological deposition (85, 86). The main mechanisms involved in α-synuclein aggregates-related microglia response are the activation of nod-like receptor (NLR) pyrin domain containing 3 (NLRP3) or caspase 1 inflammasome and nuclear factor-κB (NF-κB) signaling (87). Moreover, the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) pathway has been found to modulate the migration of microglial cells exposed to the protein (88, 89). Of note, lipopolysaccharide (LPS) and IL-1β increase the expression of α-synuclein in human macrophages (90, 91), while murine macrophages are activated by full length α-synuclein *in vitro* and *in vivo* (92, 93). Finally, the expression of α-synuclein in peripheral blood mononuclear cells (PBMCs) (94) and its modulation in PD brains support that α-synuclein may be implicated in the modulation of systemic inflammatory responses, even though its exact contribution is to be further investigated (32).

ALPHA-SYNUCLEIN IN ECs

Endothelial cells constitute a distinct cell population coating the innermost lining of blood and lymphatic vessels (95, 96). These cells are known to exhibit differential gene expression, morphology, and function across the vascular tree and organs of the body (97, 98). However, to which extent such heterogeneity impacts the endothelial dysfunctions in neurodegenerative diseases such as PD remains unclear. Cerebral ECs exert multiple functions, including the formation of the BBB, the regulation of immune cells trafficking and vascular hemostasis, and the control of cell migration and proliferation (95). In the CNS, ECs organize and maintain the BBB through anatomical and molecular interactions with neurons, pericytes, astrocytes, microglia, and perivascular macrophages in the neurovascular unit (NVU) [(99–103); **Figure 1**]. Moreover, ECs and astrocytes secrete and deposit basement membranes (BM) that provide additional barrier functions [(104); **Figure 1**]. Interestingly, studies in stroke and PD models showed that BBB disruption leads to enhanced neuroinflammation and accumulation of toxic forms of α-synuclein, which in turn could promote the progression of neuronal loss by impacting on diverse components of the BBB [(73, 105); **Figure 1**].

The expression of α-synuclein in vascular ECs supplying the brain and peripheral organs has been known for a long time (96). In the normal human brain, a gradient distribution appears to exist, where α-synuclein is present in higher levels in ECs of leptomeningeal vessels, while intra-parenchymal and capillary ECs show lower and no expressions, respectively (106). Nonetheless, the existence of such graded expression in PD, its functional relevance, and regulation have not been elucidated yet. Conversely, ECs lines, including those derived from cerebral micro-vessels, exhibit low endogenous α-synuclein levels when compared to neurons (38, 39, 106).

Interestingly, transmitted-electron microscopy studies addressing subcellular localization in ECs, identified α-synuclein near WPBs, elongated intracellular granules that contain chemokines, cytokines, and adhesive molecules which are rapidly released into the extracellular space by agonists and modulate ECs response to stimuli (38). Pathological conditions such as hypoxia, ischemia, inflammation, and oxidative stress increase α-synuclein levels, its aggregation in neurons, and to some extent in non-neuronal cells *in vivo* and *in vitro* (35, 73, 74, 90, 107–109). However, similar stimuli failed to upregulate α-synuclein levels in ECs (106, 110), supporting the need for a better understanding of the mechanisms regulating its expression in these cells. Interestingly, wild-type and mutant α-synuclein inhibit the agonist-induced-release of von Willebrand factor (vWF) and P-selectin translocation from WPBs in ECs (38). These processes enable ECs to control vascular homeostasis during inflammatory response and thrombosis [(111, 112); **Figure 2**]. Indeed, agonists such as thrombin, vascular endothelial growth factors (VEGF), histamine, and superoxide can induce an increase in intracellular calcium levels. Subsequently, calcium binds and activates calmodulin which then triggers the translocation of Ral specific guanine exchange factor (RalGDS), from cytosol to plasma membrane and activates membrane-bound RalA (a small GTPase and substrate for RalGDS) by exchanging GDP with GTP [(112); **Figure 2A**]. Afterward, the RalA-GTP interacts with and assembles exocyst, a multi-protein complex important in targeting vesicles to membranes (113), to promote the exocytosis of WPBs (**Figure 2A**). In parallel, forskolin, or epinephrine can increase cyclic adenosine monophosphate (cAMP) levels thus inducing protein kinase A activation, which also causes RalGDS membrane translocation [(38, 112); **Figure 2A**]. Upon activation of these pathways, α-synuclein binds to both RalGDS and

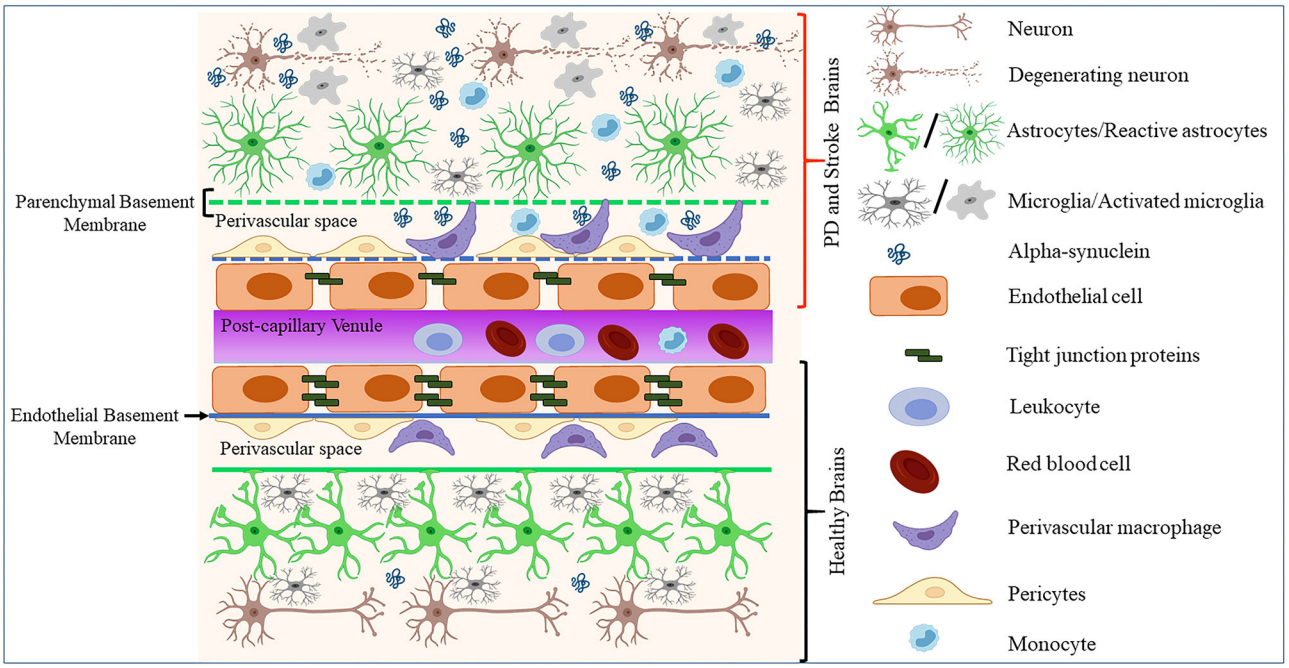

FIGURE 1 | Schematic representation of how pathological α-synuclein-accumulation occurring in PD or stroke can disrupt the physiological homeostasis of the BBB by affecting its diverse components. The BBB is composed of microvascular endothelial cells, pericytes, astrocytes, and BM components deposited by ECs (endothelial BM) and astrocytes (parenchymal BM). More recently, perivascular macrophages and vessel-associated microglia were found to play a role in the maintenance and repair of BBB whose disruption is detected in various neurological disorders including stroke and PD. This could result in BM damage (dotted lines), downregulation of TJ proteins, abnormal accumulation, and spreading of toxic forms of proteins such as α-synuclein, activation of glial cells and PVMs, and infiltration of peripheral leukocytes and monocytes, leading to neuronal degeneration.

β-arrestin, thus enhancing their interaction and inhibiting their dissociation and translocation to the plasma membrane, thereby preventing WPBs exocytosis [(38); **Figure 2B**]. Moreover, it may be also feasible to foresee that exocytosis might also be prevented by inactivation of phospholipase D (PLD) due to α-synuclein overexpression-related ER stress (114) or enhanced polymerization of actin filaments by α-synuclein [(18, 115, 116); **Figure 2B**], that by immobilizing WPBs would avoid their release.

Alpha-synuclein is present in the cerebrospinal fluid (CSF) and the blood and transported across the BBB (59, 117–120). In particular, α-synuclein can be transferred by multiple transport mechanisms including carriers such as lipoprotein receptor-related protein-1 (LRP-1) (59) or extracellular vesicles (EVs) (121). Exosome-derived α-synuclein induces oligomerization of endogenous soluble protein in recipient cells and contributes to intercellular propagation of pathology. The CSF of patients with PD show α-synuclein containing exosomes derived from various cells including microglia and exert different functions (122, 123). In line with this, erythrocyte-derived exosomes containing α-synuclein from patients with PD induce microglial activation *in vivo* and *in vitro*, thus suggesting that erythrocyte-derived extravasated α-synuclein may play a role in disease pathogenesis (121). These evidences support that further studies are needed to understand how exosome-associated physiological or pathological forms of the protein may impact on brain immune cells and ECs function and thus on BBB integrity.

Alpha-synuclein-induced inflammation might contribute first to the stimulation of rapid ECs response, which by driving the contraction of ECs, leads to the formation of gaps between them. This reshaping of ECs alters the continuous ECs layer mediating the improvement of its paracellular permeability and induces the activation of ECs. Consequently, the induction of proinflammatory molecules production and release from ECs increases the local blood flow. These events, in conjunction with the ECs layer alteration, prompt BBB dysfunction, leading to the extravasation of protein-rich exudates as well as to the recruitment and activation of circulating leukocytes, that further promote neuroinflammation (124). In particular, it may be feasible that the chronic upregulation of TNF-α and IL-1β associated with α-synuclein deposition, observed in patients with PD and animal models (82), might induce sustained activation of ECs. The consequent activation of NF-κB and activator protein 1 (AP-1) and the production of vascular cell adhesion molecule 1 (VCAM 1) and intercellular adhesion molecule 1 (ICAM 1) (124), would thus set the stage for enhanced neuroinflammation, BBB injury, and neurodegeneration (**Figure 2**). On this line, the mechanisms linking α-synuclein deposition to endothelial injury warrants further investigation.

Evidence supports that α-synuclein preformed fibrils (*pffs*) downregulate the expression of occludin and of zonula occludens

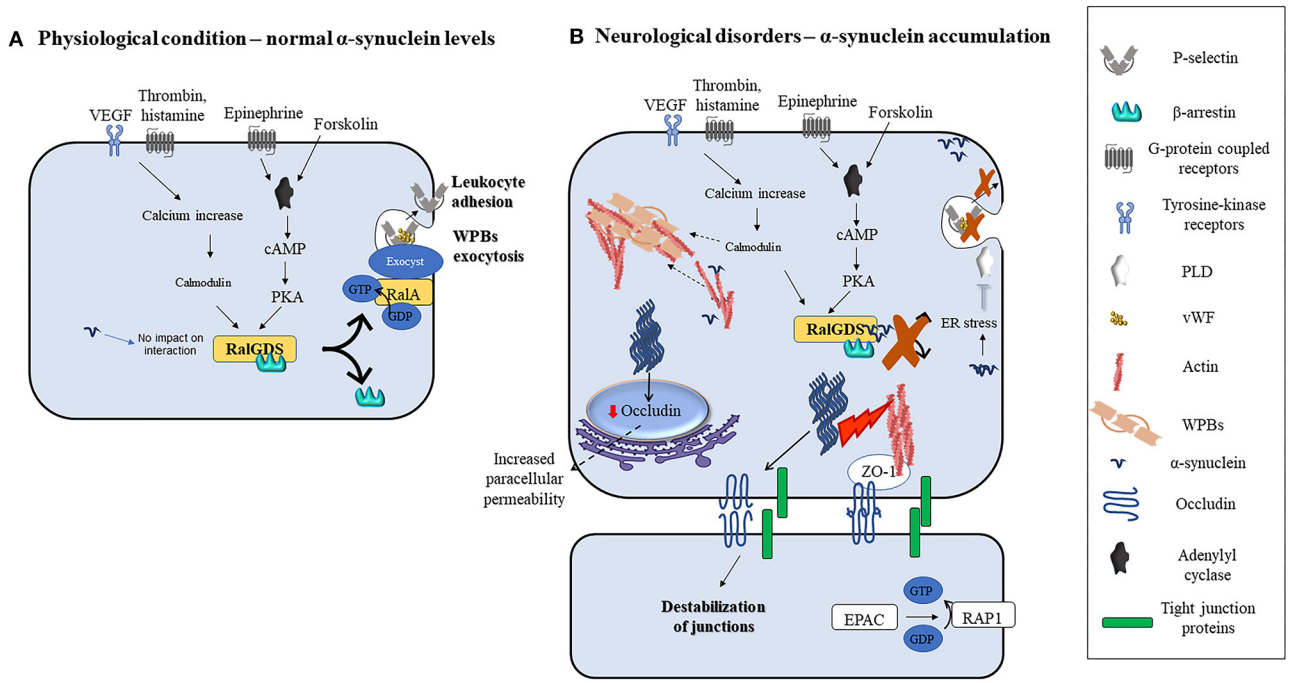

FIGURE 2 | Modulation of ECs function by α-synuclein in physiological or pathological conditions. **(A)** Various agonists bind to endothelial cells G-protein coupled receptors (thrombin, superoxide, histamine, and epinephrine) or tyrosine kinase receptors (VEGF) to activate different intracellular pathways that lead to the release of contents of WPBs and translocation of P-selectin leading to leukocyte adhesion. Thrombin, VEGF, histamine, and superoxide lead to intracellular calcium increase and calmodulin activation, while forskolin and epinephrine increase intracellular cAMP and activate PKA. Calmodulin and PKA then trigger the translocation of RalGDS from cytosol to plasma membrane that activates membrane-bound RalA by exchanging GDP with GTP. This results in WPBs exocytosis. **(B)** Alpha-synuclein inhibits the release of contents of WPBs *in vitro* by various putative mechanisms which include binding to RalGDS and β-arrestin to prevent their dissociation, and hence exchange of RalA, blocking PLD activity, and immobilization of WPBs through enhanced actin polymerization. Fibrillary α-synuclein destabilizes TJ thereby affecting ECs paracellular permeability. This may occur through the downregulation of occludin and ZO-1.

1 (ZO-1) (**Figure 2B**). As a consequence, the transport across intercellular junctions between ECs could be improved (39). However, α-synuclein *pffs* do not trigger endothelial dysfunction or release of proinflammatory cytokines from ECs in culture (39), supporting that these cells are less vulnerable to α-synuclein toxicity. On the other hand, activation of Ras homologous guanosine triphosphate phosphatase (RhoGTPases) leads to distinct effects on the ECs' barrier function depending on the type of GTPase activated (125, 126). For instance, excess activation of RhoA by thrombin or VEGF induces the formation of stress fibers which destabilizes intercellular junctions and downregulates the expression of eNOS, thereby promoting paracellular permeability and endothelial dysfunction [(126); **Figure 2**]. A recent study on a human brain-chip modeling the *substantia nigra* (SN) showed that α-synuclein fibrils can induce increased paracellular permeability (127). Interestingly, transcriptomic analysis of the ECs in the brain-chip revealed the upregulation of genes involved in inflammation, oxidative stress, autophagy, efflux system, and extracellular matrix deposition and the downregulation of genes that encode for TJ proteins (127). Conversely, the overexpression of A30P mutated α-synuclein has been found to upregulate collagen IV α2 chain (COL4A2), a major constituent of BMs *in vivo* and *in vitro* (22), further supporting that α-synuclein changes may impact the BBB integrity also by affecting this component. However, whether and how α-synuclein influences these pathways to regulate ECs functions at the BBB or the secretion and assembly of other BM elements, their degrading enzymes, or interaction with receptor proteins and other neighboring cells still needs to be addressed. Likewise, since endothelial dysfunction might, in turn, alter the transport of α-synuclein between the brain and vasculature, thus promoting its accumulation and the progression of α-synuclein pathology, studies addressing whether and how BBB dysfunction may impact PD progression could bring new insights into our basic understanding of the pathophysiology of this disorder.

Indeed, brains of patients with PD show evidence of endothelial degeneration, downregulation of TJ proteins, and even angiogenesis (39, 105, 128, 129). These changes were observed mostly in the SN, *locus coeruleus* (LC), and *caudate putamen* (CP), brain regions where α-synuclein-induced degeneration is prominent, and to a lesser extent in the cerebral cortex (105, 128, 129). Moreover, pathological alterations in the capillary BM including collagen deposition and thickening are evident in PD brains (12, 128, 130). It is thus plausible to speculate that such changes may reduce the efficiency of the exchange of molecules between the brain and vasculature, rendering neurons vulnerable to oxidative stress and accumulating cellular waste products.

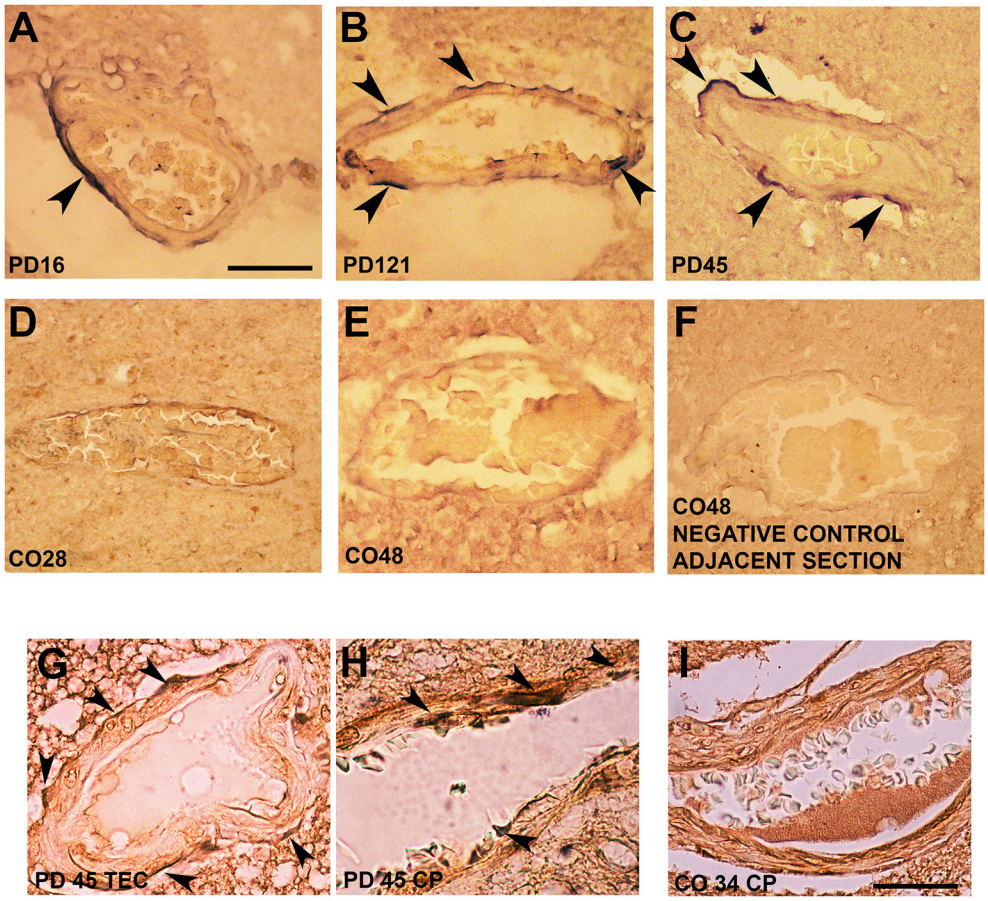

FIGURE 3 | Alpha-synuclein perivascular immunoreactivity in *postmortem* sections from sporadic patients with PD. For these experiments, sections from three patients with PD (PD16, disease duration 18 years; PD45, disease duration 19 years; and PD121, disease duration 4 years) and three healthy controls (CO28, CO34, and CO48), kindly supplied by the Parkinson's UK Brain Bank, were analyzed. Briefly, sections were treated for antigen retrieval with 10 mM sodium citrate (20′ at 95°C) and 10% formic acid (15′ at RT). After 1 h incubation at room temperature (RT) with blocking solution (2% w/vol bovine serum albumin, 3% vol/vol normal goat serum, 0.3% Triton X-100 diluted in PBS 0.1 M pH 7.4) the 5-μm slices were subjected to either single α-synuclein (Sin211 MA5-12272 Thermo Fisher Scientific, Waltham, USA; dilution 1:500) or double laminin α2 (4H8-2, abcam ab11576; dilution 1:100)/α-synuclein (Sin211, MA5-12272 Thermo Fisher; dilution 1:500) immunolabeling according to previously described protocols (137, 138). Single α-synuclein immunopositive signal was revealed by Blue Alkaline Phosphatase (Vector Laboratories, Burlingame, CA) acquired by using a 40X objective, while for double immunolabeling laminin α2 was revealed by brown 3,3-diaminobenzidine (DAB) and α-synuclein by violet (Nickel supplemented) DAB (Vector laboratories) and acquired by a 100X objective. All the images were acquired by using an inverted light microscope (Olympus BX41; Olympus, Milan, Italy). **(A–C)** Representative images of perivascular α-synuclein immunolabeling (blue, arrows) in the TEC of three sporadic PD cases (PD 16, PD 121, and PD 45). **(D–F)** Images from the TEC of two of the healthy controls analyzed (CO28 and CO48). **(D,E)** The absence of α-synuclein immunolabeling in control brains, while **(F)** shows a representative image from a negative control for the immunostaining performed without the addition of the primary antibody on an adjacent section of CO48. **(G,H)** Representative images showing the presence of α-synuclein violet immunolabeling at the outer (**G**, arrows) and inner (**H**, arrows) side of laminin α2-positive perivascular BM in the brain of a sporadic patient with PD (PD45). Images are representative of the TEC **(G)** and CP **(H)**. **(I)** Representative image showing the absence of α-synuclein accumulation around laminin α2-immunolabeling in the proximity of a vessel of the CP of a healthy control (CO34). Scale bar: **(A–F)** 40 μm; **(G–I)** 25 μm.

Angiogenesis is a well-recognized adaptive response to cerebral hypoxia or ischemia and is regulated by BM proteins and their integrin receptors (131). Interestingly, the integrin receptor αvβ is upregulated in angiogenic vessels (131, 132) and in cerebral vessels of patients with PD and incidental LB disease (iLBD) (129), suggesting that the immature nascent vessels generated in PD brains, could contribute to neuroinflammation by facilitating the infiltration of peripheral immune cells and inflammatory or toxic factors (129). Consistently, co-localization of areas of leakage of an intravascular tracer with β3 integrin-expressing new vessels, indicating the presence of both angiogenesis and compromised BBB, has been observed in toxin-induced animal model of PD (132). Based on Braak's PD staging (56), patients with iLBD may represent an early disease stage where LB is restricted to LC and SN (129). Therefore, the presence of angiogenesis in patients with iLBD and PD supports that α-synuclein related vascular dysfunction might precede or/and contribute to the progression of neuroinflammation and neurodegeneration. This is further substantiated by findings showing that α-synuclein-related angiogenesis and downregulation of TJ proteins are not necessarily related to inflammation (39, 129). Indeed, recent

findings indicate that dysfunction in BBB accompanied by pathological activation of pericytes precedes the onset of neuronal degeneration in a mouse model of PD (133), thus supporting that vascular dysfunction may be an early pathogenic events leading to neuronal damage. Furthermore, VEGF plays a protective role in PD through a direct effect on dopaminergic neurons (134) or via the canonical VEGF receptor (VEGFR2) pathway (135). VEGF released by activated astrocytes and microglia acts in a paracrine fashion to modulate ECs structure and function both in PD and DLB patients and in animal models of PD (136). This notwithstanding, whether α-synuclein is involved in the upregulation of VEGF in ECs remains to be investigated.

Interestingly, in *postmortem* sections of the trans-entorhinal (TEC) cortex (**Figures 3A–C**) and CP (not shown) from sporadic patients with PD, we observed perivascular accumulation of α-synuclein immunoreactivity (in blue) in correspondence of some vessels. Conversely, the brains of healthy or negative controls did not exhibit this feature (**Figures 3D–F**). By double immunolabeling of laminin α2 (brown) and α-synuclein (violet), we found that in the PD brains, α-synuclein-positive perivascular staining could be identified either in the outer (**Figure 3G**) and inner (**Figure 3H**) sides of the perivascular basement membrane, while in control brains, it did not show α-synuclein positivity in the proximity of laminin α2 staining (**Figure 3I**).

Although further studies are needed to corroborate whether and how α-synuclein deposition affects these cells, these findings, when coupled to the aforementioned noxious effects exerted by pathological α-synuclein on ECs, support that perivascular accumulation of the protein, by inducing ECs activation may compromise brain vessels integrity exacerbating astrocyte and microglia activation thus promoting neurodegeneration and BBB disruption (**Figure 4**).

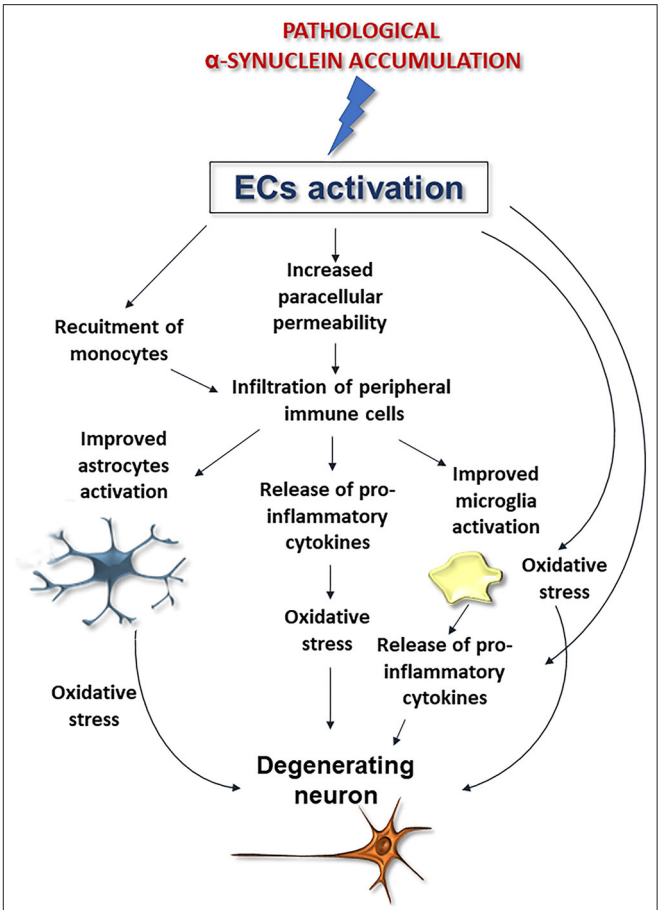

FIGURE 4 | Possible downstream effects of pathological α-synuclein-induced activation of ECs on microglia, astrocytes, and neurons. Alpha-synuclein accumulation-induced ECs activation could promote BBB disruption and peripheral immune cell infiltration, improve microglia and astrocytes activation and exacerbate oxidative stress, thus promoting neuronal degeneration.

ALPHA-SYNUCLEIN IN BAMs AND OTHER PERIVASCULAR CELLS

Border-associated macrophages are a subset of CNS myeloid cells (macrophages) that like microglia originate prenatally in the yolk sac (139), invade the brain during the early prenatal period, and localize in the choroid plexus, perivascular, and leptomeningeal spaces. BAMs form stable populations with the sole exception of the choroid plexus macrophages that exchange with peripheral monocytes (139, 140). Indeed, BAMs can be anatomically distinguished into perivascular macrophages (PVM), meningeal macrophages (MAM), and choroid plexus associated macrophages (CPM) (139, 140). In healthy brains, PVMs are involved in the regulation of BBB permeability and phagocytosis of pathogens but can also promote the entrance of peripheral immune cells into the brain (102). Under pathological conditions such as cerebral amyloid angiopathy (CAA), AD (141) and PD (141), PVMs can participate in the clearance of toxic amyloid-β and α-synuclein. Consistently, the depletion of PVMs using clodronate-containing liposomes in a mouse model of PD resulted in the increased expression of VCAM 1, the infiltration of T cells, and the propagation of α-synuclein pathology (142). Moreover, in rats that underwent transient ischemia followed by reperfusion injury, BAMs were involved in promoting peripheral immune cell infiltration and vascular permeability without impacting the extent of ischemic damage, thus suggesting that additional studies are needed to fully understand the modulatory role of these cells in cerebrovascular dysfunctions and neuroinflammation (13).

Border associated macrophages are involved in immune surveillance and support the entrance of peripheral immune cells into the CNS under pathological conditions (143, 144). In rodents, monkeys, and humans, PVMs express the mannose receptor CD206 (145) and the scavenger receptor CD163, which under physiological conditions is expressed on tissue macrophages, with the exception of microglia and some monocytes (146–148). Recent research reports, dissecting the molecular signature of brain macrophages in mice at the single-cell level, reported a clear segregation of BAMs from microglia, identified a BAMs core gene signature, and even showed heterogeneity within BAMs (149–152). In stroke animal models

and patients, a unique transcriptional signature of BAMs, their local proliferation and migration in the brain parenchyma, have been detected (14).

It is now believed that BAMs play a role in immune function, BBB integrity, and lymphatic clearance (139, 140). Currently, the identification of BAMs mainly relies on the use of anatomical studies aimed at disclosing their localization in the brain thanks to the use of few reliable molecular markers (139, 140). However, this approach is not applicable in the presence of inflammatory conditions or tissue injury when peripheral monocytes/macrophages enter the brain and reside in the same location and express similar molecular markers (139). Despite these limitations, remarkable progresses have been made to fully characterize and understand their role in the normal and diseased brain.

Alpha-synuclein is expressed by microglia and peripheral monocytes/macrophages in a lower amount compared to neurons (32), but the expression in BAMs has not been described yet. Since BAMs share similar ontogeny and molecular and immunologic characteristics with microglia (139, 140), they might exhibit analogous changes and activation states to α-synuclein stimulation (153, 154). Indeed, it has been described that BAMs and microglia display multiple similarities such as the expression of myeloid-specific markers. Among them, ionized calcium-binding adaptor molecule 1(Iba1), F4/80 (mouse) or EMR1 (human), chemokine receptors, scavenger receptors, receptor tyrosine kinases, Integrins, pattern recognition receptors (PRRs), and cytokines receptors (155). α-synuclein is known to induce inflammatory response and migration of microglia (32, 89, 156). For instance, previous studies showed that α-synuclein induces NOX2 activation in microglia by binding to toll-like receptor 2 (TLR-2) and CD11b leading to microglia-mediated neuronal toxicity (157). Similarly, α-synuclein binds to TLR-4 and activates NF-kB signaling which then induces a selective autophagy pathway named synucleinphagy and release of exosomes containing the protein, thus contributing to the intercellular spread of α-synuclein pathology (154).

Interestingly, monomeric α-synuclein can also impact microglia polarization by conferring an anti-inflammatory profile to these cells through the interaction with extracellular signal-regulated kinase (ERK) and the recruitment of the ERK/NF-κB, and peroxisome proliferator-activated receptor γ (PPARγ) pathways (158). It may thus be feasible that these pathways may also be activated upon exposure of BAMs to α-synuclein.

It is worth mentioning that pericytes can also play a role in the formation, maintenance, and regulation of BBB (159). Additionally, pericytes (60) and astrocytes (160) can mediate the transfer of α-synuclein between cells of the NVU, suggesting a possible role of non-neuronal cells in α-synuclein pathology spreading in PD (161). *In vitro* studies also showed that α-synuclein activates pericytes which in turn release proinflammatory mediators that can mediate BBB dysfunction (162). Early pericyte activation associated with BBB leakage has been recently described in a human α-synuclein overexpression-based mouse model of PD (133), thus further supporting that vascular pathology can constitute a relevant pathophysiological aspect of PD.

Peripheral immune cells such as lymphocytes have also been involved in the pathogenesis of PD. Indeed, studies in the brains of patients with PD and animal models showed that T cells with upregulated expression of the ICAM 1 receptor lymphocyte function-associated antigen-1 (LFA1) can promote leukocyte infiltration (163). Alpha-synuclein-specific T-cell reactivity has been found to be higher in early PD while decreasing in patients with late-stage disease (164). When considering the increase of α-synuclein in animal models of stroke (73), where Treg cells interact with ICAM1 on inflamed microvessels and platelets promoting vascular dysfunction (165), this evidence suggests that the accumulation of α-synuclein occurring following brain ischemia could very well-boost these pathogenic processes.

Several studies showed that α-synuclein aggregates can be detected in reactive astrocytes in the brains of patients with PD and animal models (33) suggesting a role of these cells in the clearance and /or spreading of α-synuclein toxicity in the brain parenchyma and NVU. Even though the role of endogenous α-synuclein in astrocytes remains to be fully explored, in disease states, α-synuclein activates astrocytes by interacting with PRRs such as TLR-4 (33). Activated astrocytes can in turn uptake and degrade the protein via the endosomal-lysosomal pathway and contribute to non-cell autonomous degeneration (166, 167).

Recent evidence suggests that α-synuclein can be removed from the brain via extracellular space drainage pathway which includes glymphatic transport and meningeal lymphatic system (61), whose reduction lead to the accumulation of toxic forms of amyloid-β in the brain parenchyma of AD rodent models (168–170). Similarly, a recent study in a transgenic PD mouse model overexpressing human A53T mutated α-synuclein showed that blockage of the deep cervical lymph node reduces glymphatic transport of an intraventricular tracer and promotes the accumulation of α-synuclein and its aggregation in SN, thus leading to the progression of α-synuclein pathology (61).

Taken together, impairment in these systems results in the accumulation of toxic proteins in the brain and contributes to the progression of neurodegenerative diseases. The close association of BAMs to perivascular and lymphatic drainage systems in the brain when coupled to the detection of α-synuclein aggregates in the perivascular space of a PD mouse model (61) supports that understanding of whether and how these cells contribute to the clearance of α-synuclein along these pathways deserves *ad-hoc* investigation.

In addition to this, it is plausible that the increase in α-synuclein levels observed following ischemia and spinal cord injury (35, 73, 74, 171) could result in a chemotactic gradient for microglia migration and activation (89) contributing to brain damage. Consistently, inhibition of α-synuclein induction following ischemia or spinal cord injury reduces secondary neuronal injury, inflammatory response, and improves neurological outcomes (171, 172). Although, it is known that juxta vascular microglia play a divergent role in repairing vascular injuries following an insult or systemic inflammation (173–175), whether α-synuclein modulates these cells or BAMs remains to be clarified.

ALPHA-SYNUCLEIN ROLE IN MODULATING BBB CELLS INTERACTION

In the normal and diseased brain, ECs communicate with neurons, microglia, pericytes, and astrocytes to regulate vascular function (176). More importantly, the interaction of microglia with ECs can exert divergent roles in regulating BBB integrity (175). In co-cultures of ECs and neurons, α-synuclein fibrils resulted in endothelial dysfunction, but this effect was not observed in ECs monocultures (39), supporting that neuronal-ECs crosstalk at the NVU may be perturbed by pathological α-synuclein.

CD200, a transmembrane protein found to be expressed in neurons, astrocytes, oligodendrocytes, and ECs, transduces signal via its receptor (CD200R), expressed on myeloid cells including microglial and BAMs (177). CD200-CD200R and C-X3-C motif chemokine ligand 1 (CX3CL1)-C-X3-C motif chemokine receptor 1 (CX3CR1) signaling between neurons and microglia helps to maintain microglia in the resting state (178). Inactivation of the transmembrane glycoprotein CD200R in microglia of a toxin-induced PD mouse model results in increased activation of these cells, release of proinflammatory cytokines, loss of dopaminergic neurons in the SN, and behavioral deficits (179). Similarly, monocyte-derived macrophages (MDMs) from patients with PD show dysregulation in CD200R signaling (180).

On the other hand, M2 macrophages express pro-angiogenic factors such as VEGF and fibroblast growth factor 2 (FGF2), that by activating their receptors (VEGFR2 and FGFR) promote angiogenesis and neuronal survival (181). *In vitro* studies have shown that microglia maintains ECs in a resting state by secreting transforming growth factor-beta (TGF-β), an anti-inflammatory cytokine, while the proinflammatory TNF-α induces ECs proliferation (182). However, whether this kind of communication occurs also between ECs and BAMs or is influenced by α-synuclein still remains to be elucidated.

Secreted toxic species of α-synuclein are known to bind to various cell surface receptors in adjacent cells and activate several intracellular pathways leading to synaptic dysfunction, neurodegeneration, and inflammation (183). On this line, α-synuclein binds to TLR-2, TLR-4, and CD11β integrin to activate NF-kB signaling and assembly of NLRP3 inflammasome in microglia (37, 154, 157). It is thus plausible that various receptors for α-synuclein might exist in different cells, including ECs, and that the protein may regulate their intracellular activities or their crosstalk within neighboring cells such as BAMs.

CURRENT GAPS AND FUTURE PERSPECTIVES

While α-synuclein associated vascular dysfunction is evident in PD (11, 128), most of the studies aimed at understanding the physiological role of the protein in ECs have been performed by overexpressing the protein through transgene expression (38, 39). Therefore, the role of extracellularly released α-synuclein on these cells still needs to be extensively explored. Furthermore, it is not yet established whether ECs respond to various pathogenic stimuli by regulating α-synuclein level or its transport across the BBB. Future studies exploiting improved models might overcome these limitations.

It is now clear that α-synuclein-associated inflammation contributes to the pathophysiology of PD (10) but research on whether or how α-synuclein modulates the function of macrophages in the brain has been mostly focused on microglia. As a result, our knowledge on the role of α-synuclein in other brain resident macrophages such as BAMs or other perivascular cells is poor. Similarly, despite the presence of studies indicating interplay between ECs and microglia (175), the interplay between perivascular cells and ECs has been scarcely studied. For instance, since recent evidence showed that BAMs and microglia can acquire distinct genetic and molecular phenotypes early in development (152), studying whether α-synuclein plays a role in modulating the signaling pathways mediating the crosstalk between BAMs and ECs could bring novel and significant insights for understanding the biological basis of neurological disorders such as PD or stroke. Similarly, though α-synuclein transfer between cells and across BBB interfaces has been established (59, 154), whether and how BAMs are involved in this event also deserves further investigations.

A deeper understanding of the role of physiological and pathological forms of α-synuclein in the modulation of BAMs and ECs or their interplay may also greatly aid the identification of novel therapeutic targets for stroke or neurodegenerative synucleinopathies.

AUTHOR CONTRIBUTIONS

TB and GF wrote and revised the article and prepared the figures. TB performed immunostaining. FL revised the article and was involved in image acquisition. AB, MP, and SM did a critical revision of the article. All authors contributed to the article and approved the submitted version.

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GLOSSARY

AD, Alzheimer's disease; AP-1, activator protein 1; BAMs, border associated macrophages; BBB, blood–brain barrier; BM, basement membrane; CAA, cerebral amyloid angiopathy; cAMP, cyclic adenosine monophosphate; CD200R, CD200 receptor; CNS, central nervous system; COL4A2, collagen IV alpha 2 chain; CP, *caudate putamen*; CPM, choroid plexus associated macrophages; CSF, cerebrospinal fluid; CX3CL1, C-X3-C motif chemokine ligand 1; CX3CR1, C-X3-C motif chemokine receptor 1; DAB, 3,3-diaminobenzidine; DLB, dementia with LB; ECs, endothelial cells; ER, endoplasmic reticulum; ERK, extracellular signal-regulated kinase; EVs, extracellular vesicles; FGF2, fibroblast growth factor 2; FGFR, FGF2 receptor; GCI, glial cytoplasmic inclusions; Iba1, ionized calcium-binding adaptor molecule 1; ICAM 1, intercellular adhesion molecule 1; iLBD, incidental LB disease; IL, interleukin; iPSC, induced pluripotent stem cell; LB, Lewy bodies; LC, *locus coeruleus*; LFA, lymphocyte function-associated antigen-1; LPS, lipopolysaccharide; LRP-1, lipoprotein receptor-related protein-1; MAM, meningeal macrophages; MDMs, monocyte-derived macrophages; MSA, multiple system atrophy; NAC, non-amyloid component; NADPH, nicotinamide adenine dinucleotide phosphate; NF-κB, nuclear factor-κB; NK, natural killer; NLR, nod-like receptor; NLRP3, NLR pyrin domain containing 3; NOX, NADPH oxidase; NVU, neurovascular unit; PBMCs, peripheral blood mononuclear cells; PBS, phosphate buffer saline; PD, Parkinson's disease; *pffs,* preformed fibrils; PLD, phospholipase D; PPARγ, peroxisome proliferator-activated receptor γ; PRRs, pattern recognition receptors; PVM, perivascular macrophages; RalGDS, Ral specific guanine exchange factor; RhoGTPases, Ras homologous guanosine triphosphate phosphatase; RT, room temperature; SN, *substantia nigra;* TEC, trans-entorhinal cortex; TGF-β, transforming growth factor-beta; TJ, tight junction; TLR-2, toll-like receptor 2; TLR-4, toll-like receptor 4; TNFα, tumor necrosis factor alpha; VCAM 1, vascular cell adhesion molecule 1; VEGF, vascular endothelial growth factors; VEGFR2, VEGF receptor; vWF, Willebrand factor; WPBs, Weibel-Palade bodies; ZO-1, zonula occludens 1.

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REVIEW
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Dwellers and Trespassers: Mononuclear Phagocytes at the Borders of the Central Nervous System

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The central nervous system (CNS) parenchyma is enclosed and protected by a multilayered system of cellular and acellular barriers, functionally separating glia and neurons from peripheral circulation and blood-borne immune cells. Populating these borders as dynamic observers, CNS-resident macrophages contribute to organ homeostasis. Upon autoimmune, traumatic or neurodegenerative inflammation, these phagocytes start playing additional roles as immune regulators contributing to disease evolution. At the same time, pathological CNS conditions drive the migration and recruitment of blood-borne monocyte-derived cells across distinct local gateways. This invasion process drastically increases border complexity and can lead to parenchymal infiltration of blood-borne phagocytes playing a direct role both in damage and in tissue repair. While recent studies and technical advancements have highlighted the extreme heterogeneity of these resident and CNS-invading cells, both the compartment-specific mechanism of invasion and the functional specification of intruding and resident cells remain unclear. This review illustrates the complexity of mononuclear phagocytes at CNS interfaces, indicating how further studies of CNS border dynamics are crucially needed to shed light on local and systemic regulation of CNS functions and dysfunctions.

Keywords: macrophage cell, meninges, CNS inflammation, cell trafficking, choroid plexus

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INTRODUCTION

The borders of the central nervous system (CNS) parenchyma are complex structures which maintain organ homeostasis through distinct anatomical specializations. These border areas halt the transit of potentially harmful trespassers contributing to the establishment of a relatively immune-privileged milieu within the CNS parenchyma (1). At the same time, these functional barriers host

Abbreviations: CNS, central nervous system; SAS, subarachnoid space; BCSFB, blood-cerebrospinal fluid barrier; BBB, blood-brain barrier; ChP, choroid plexus; lpM, resident leptomeningeal macrophage; pvM, resident perivascular macrophage; ChPM, resident stromal choroid plexus macrophage; MdM, monocyte-derived macrophage; BAM, barrier associated macrophage; MS, multiple sclerosis; EAE, experimental autoimmune encephalomyelitis; ROS/RNS, reactive oxygen and nitrogen species; SCI, spinal cord injury; TBI, traumatic brain injury; AD, Alzheimer's disease; Aβ, amyloid-β; PD, Parkinson's disease; DAMPs, damage-associated molecular patterns.

an extensive variety of yolk sac- and bone marrow-derived myeloid cells, cellular dwellers which are an integral part of the historically overlooked CNS immune capabilities. Altogether, CNS interfaces are fundamental participants in CNS functions and defense mechanisms, as well as contributing to the overall integration of the CNS with the rest of the organism (2–4).

While an increasing body of research is finally dedicating attention to CNS borders and their cellular components, surprisingly much remains to be investigated and understood (5–7).

In this review, we will illustrate the functions and migratory routes of monocyte-derived and tissue-resident macrophages, the immune cells that most densely populate CNS interfaces during homeostasis and upon damage and inflammation (8).

BARRIER-ASSOCIATED DWELLERS: LOCATION AND HOMEOSTATIC FUNCTION OF CNS MACROPHAGES

CNS borders contain functional barriers separating the CNS parenchyma from peripheral circulation at the level of I- the leptomeningeal/subpial vasculature within the subarachnoid space (SAS), II- the blood-cerebrospinal fluid barrier (BCSFB) of the choroid plexus (ChP) and of the arachnoid mater, and III- the blood-brain/spinal cord barrier (BBB) within parenchymal vessels. As an exception to this rule, circumventricular organs lining the brain ventricles and possessing endocrine functions lack a BBB (9). Furthermore, the CNS parenchyma is protected by the astrocytic glia limitans which envelops perivascular and meningeal surfaces (10) allowing a double layer of separation between parenchymal cells and peripheral circulation (11–13).

CNS interfaces harbor populations of tissue-resident macrophages often referred to as CNS-associated macrophages or barrier-associated macrophages (BAMs, **Figure 1**) (14–16). Once mistakenly believed to derive from adult bone marrow progenitors (17, 18), most BAMs originate in the yolk sac during embryonic development and stably populate the respective niches by self-renewal throughout adulthood (14), as previously shown for microglia (19). The complex development of BAMs and microglia, deriving from distinct yolk sac-derived progenitor lineages (20), has been extensively reviewed in the last years (7, 21). Sharing high expression of fractalkine receptor (CX3CR1) and a long half-life, compared to circulating monocytes, BAMs and microglia have been often collectively studied as CNS-resident phagocytes (22), at least until the recent discovery of microglia-specific genes and related targeted transgenic approaches (15, 23).

Compared to microglia, BAMs share universally upregulated genes linked to blood vessel development, lipid and cholesterol metabolism, immune response and antigen presentation (16). In addition to the core genes *Apoe*, *Ms4a7*, *Ms4a6c*, *Tgfbi* and *Mrc1* (16), *Dab2*, *F13a1*, *Mgl2*, and *Pf4* have been recently proposed as BAM identifiers (24).

Not surprisingly, BAMs also express signature macrophage markers such as integrin αM (CD11b), Aif1 (Iba1), receptor for macrophage-colony stimulating factor (Csf1R), and F4/80 (25), the latter, however, at lower levels compared to activated macrophages and circulating monocytes (26). Expression of the adhesion molecule CD44 is negligible and can thus be used to distinguish BAMs from $CD44^+$ blood-borne macrophages within the CNS (26). Interestingly, some BAMs express the gene encoding for the T cell receptor β, although its function remains unknown (26).

While BAMs at the BBB and within the leptomeninges are solely yolk sac-derived, dura mater, and ChP interfaces harbor a mixed resident population including blood-borne monocyte-derived cells during steady state (14, 16). Novel techniques such as mass cytometry (through CyTOF) and single-cell RNA sequencing (scRNAseq) have indeed revealed a surprising heterogeneity of BAMs (16, 26–28), despite the intrinsic limitations of these approaches due to the use of predefined markers (mass cytometry) and under-representation of lowly expressed genes (scRNAseq) (16, 29).

In the next chapters, we will illustrate how BAM complexity is inherently linked to the different anatomical locations that these cells inhabit (30). A summary of cellular locations, origin and known markers in mice and humans can be found in **Tables 1** and **2**.

Resident Perivascular Macrophages

The low pinocytic endothelial cells forming parenchymal CNS vessels possess specialized features constituting the BBB, a relatively impermeable diffusion barrier (81, 82). On the parenchymal side, astrocytic end-feet form the glia limitans to offer a second functional barrier protecting the CNS parenchyma. This astrocytic layer appears impermeable to immune cells (13) but does not form tight junctions during homeostasis and allows movement of low-molecular weight tracers (83). Together, this multilayered border limits trafficking of circulating immune cells and controls the selective exclusion of harmful substances from the CNS parenchyma as well as the intake of water, chemicals, and other molecules (3).

First described in the early 1980s as "granular pericytes" (84), perivascular macrophages (pvMs) reside between the endothelial and glia limitans basement membranes of CNS vessels (excluding capillaries and small arterioles) located in basal ganglia and white matter (85–87). PvM distribution remains, however, controversial, with recent work reporting similar densities of pvMs in peri-arteriolar and peri-venous space of the mouse brain (88).

Given their strategic location, pvMs are proposed to mediate passage of information between the CNS and the periphery (4) and to regulate lymphocyte immunesurveillance (89, 90). Indeed, pvMs express MHC class II and co-stimulatory molecules (35) and secrete cytokines and chemokines, which affect the local microenvironment upon sensing damage or inflammation (8). Moreover, pvMs help to maintain the well-being of the endothelial wall and to contribute to the regulation of vascular permeability (91, 92). In line with the physiological function of the perivascular space (93), PvMs participate in CNS waste clearance (94, 95) displaying a high endocytic rate that can be exploited to mark these cells *in vivo* (94, 96–98). PvMs can also phagocytose tracers injected in the parenchyma, which demonstrates their ability to sample outflowing CNS interstitial fluids (12). Altogether, given the influence of pvMs on vascular smooth muscle cells (88) and

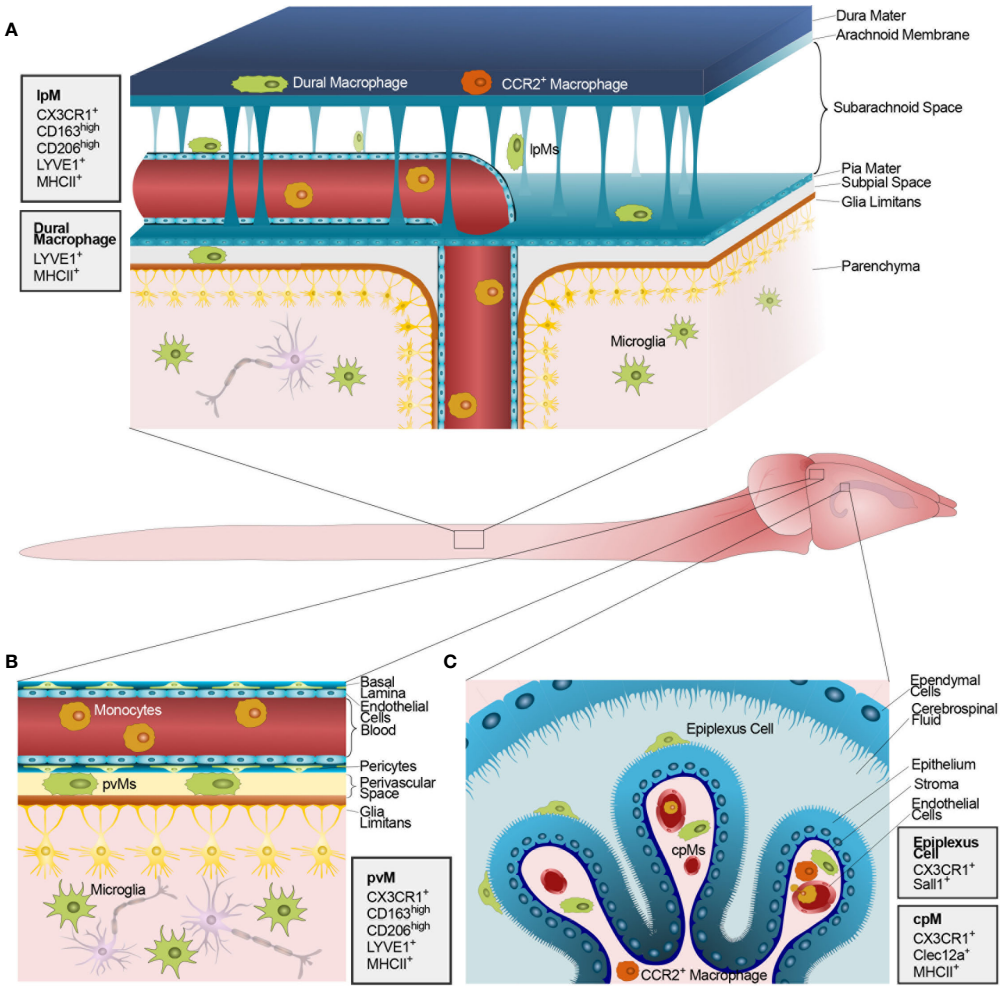

FIGURE 1 | Macrophages populating the CNS barriers and parenchyma at steady state. The figure shows the mouse CNS and, in the magnified inlets, schematic representations of the anatomical CNS interfaces containing functional barriers. **(A)** The mouse meninges including (top to bottom) the dura mater, the impermeable arachnoid mater, the SAS, the pia mater, the astrocytic glia limitans, and, finally, the CNS parenchyma. The dura mater is populated by both yolk sac-derived (green) and blood-borne CCR2$^+$ macrophages (orange). Conversely, the SAS, the subpial space and the CNS parenchyma host solely long-lived yolk sac-derived lpMs and microglia, respectively. **(B)** Schematic representation of the perivascular space at the level of post-capillary venules within the CNS parenchyma. The perivascular space hosts yolk sac-derived pvMs between a layer composed of endothelial basal lamina and pericytes and a parenchymal basal lamina. Endothelial cells forming the blood vessel are linked by tight junctions thus constituting a BBB. On the parenchymal side, astrocytic end-feet collectively form the glia limitans vascularis. **(C)** Schematic representation of the ChP within a CSF-filled brain ventricle lined by ependymal cells. On the apical side of the ChP epithelial cells, resident epiplexus cells are shown. ChP epithelial cells are linked by tight junction thus constituting a functional BCSFB. The ChP stroma hosts a combination of yolk sac-derived cpMs (green) and blood-borne CCR2$^+$ macrophages (orange) extravasated from stromal vessels lacking a BBB. Monocytes circulating within vascular lumens are shown in yellow. Next to each panel, gray boxes illustrate the main protein markers identifying CNS-resident macrophages in their distinct anatomical compartments.

the importance of pvMs on peri-arterial drainage (99), these cells appear key players in CNS fluid dynamics.

Morphologically, pvMs are compact elongated cells displaying continuous movement of cell body and protrusions (14, 100). Homeostatic pvMs are a transcriptionally homogeneous population (27). Compared to monocytes and microglia, pvMs are characterized by high expression of *Cd163* (35), a pattern recognition receptor (PRR) recognizing hemoglobin (101), *Mrc1* (CD206), a PRR responsible for scavenging circulating glycoproteins (102), and *Cd36*, a scavenger receptor implicated in efferocytosis (14, 36). Mass cytometry revealed that these cells, similarly to other BAMs, are also highly positive for CD38 (15), an ecto-enzyme with metabolic functions (98).

Earlier reports, likely affected by the technical challenge of distinguishing dendritic cell (DC) from BAMs (15), indicated expression of DC markers such as CD11c and DC-SIGN in pvMs (103). Functionally and ontogenically separate from BAMs, CNS-associated DCs are described elsewhere (16, 27, 28).

Resident Leptomeningeal Macrophages

The cerebrospinal fluid (CSF)-filled SAS regulates CNS fluid, pathogen, and immune cell dynamics (104) and hosts several types

TABLE 1 | The table indicates the main RNA and protein markers described for macrophage populations in the distinct CNS compartments in mice at steady state (homeostasis) and in different disease model.

Murine Models	Circulating Monocytes	BAMs (general markers)	lpMs	pvMs	cpMs	MdMs
Location →	**Blood**	**CNS borders**	**Leptomeninges**	**Perivascular Spaces**	**Choroid Plexus**	**CNS**
Origin → Homeostasis	**Bone Marrow** Inflammatory cells: LY6C^{high} CCR2^{+} CX3CR1^{low} (31) Patrolling cells: LY6C^{low} CCR2^{low} CX3CR1^{high} (31) Shared markers: CSF1R, GM-CSFR, PECAM-1, β2, αM integrins (32)	**Yolk Sac** *Apoe*, *Ms4af*, *MS4a6c*, *Tgfbi*, *Mrc1* (16) *Dab2*, *F13a1*, *Mgl2*, *Pf4* (24) CX3CR1 (27) CD11b, IBA1, CSF1R, F4/80 (25)	**Yolk Sac** *Pf4*, *Cbr2*, *Ms4a7*, *Stab1*, *Fcrls*, *Siglec1* (27) *P2rx*, *Egfl7*, *Clec4n*, *Clec10a*, *Folr2*, *Lyve1* (16) Certain populations: *Cxcl2*, *Nfkbiz* (27) CD163^{high} CD206^{high} (33–34) SAS LYVE1^{low}MHCII^{high} Pial LYVE1^{high}MHCII^{low} (15) CX3CR1^{low} LYVE1^{+} CD38^{+} (15, 16) **Dural Ms** **Yolk Sac and Bone Marrow** Lyve1^{low}MHCII^{high} (majority) Lyve1^{low}MHCII^{+} (minority) (15)	**Yolk Sac** *Mrc1*, *Ms4a7*, *Cbr2*, *Pf4*, *Stab1*, *Lyve1* (27) MHC-II (35) CD163^{high} (35) CD36 (14, 36) CD38 (15)	**Bone Marrow/Yolk Sac** *Mrc1*, *Ms4a7*, *Pf4*, *Stab1*, *Cbr2*, *Fcrl* (27) *Lilra5*, *Ttr* (16) Kolmer´s Epiplexus Ms: *Sall1*, *Cst7*, *Gm1673*, *Clec7a* (16) LYVE1^{+} MHCII ^{negative} LYVE1$^{negativ\ e}$MHCII^{+} LYVE1^{+} MHCII^{+} (15) CCR2 (16, 37) CD163^{+}MHCII^{+} (38, 39) Bone marrow derived resident Ms: MHCII^{low} Yolk sac derived resident Ms: MHCII^{high} (16)	**Bone Marrow**
EAE		CD11b, CSF1R, CD163, CD206 (21) MHCII, CD44, PDL1, CD117, SCA-1 (15)	*Ccl5*, *H2-Ab1*, *H2-Aa*, *H2-Eb1*, *Cd74* (27) LYVE-1 (27) IBA1^{high} (40)	*Ccl5*^{high} *Cd74*^{high} *Lyve1*^{low} *Ctsd*^{low} (27, 41) OX6, SILK6, CD40, CD80, CD86 ICAM-1, VCAM-1, CCL2, CCL3 (42)	*Ctss*, *Il1β*, *S100a9*, *S100a8*, *Ngp* (27)	*Mrc1*, *Fn1*, *Cd44*, *Mertk*, *Cd206* (27) *Saa3+*, *Cxcl10+* (43) *C1qa*, *C1qc* (44) CCR2^{+} Ly6C^{high} (44) MMP2, MMP9 (45, 46) CD44 (26) iNOS, Arginase-1 (44) F4/80^{high} (26) ChP MdMs: *Cd209*, *MertK* (27) CD74, LY6C^{high} (27), CCR2 (37)
TBI/SCI Models	LY6C^{hi} CX3CR1^{low}CCR2^{hi} LY6C^{lo} CX3CR1^{high}CCR2^{lo} (47)		LYVE1^{+} (48)	LYVE1^{+} (48)		CD163^{+} HO-1^{+} (49) CCR2^{+} (50)
PD Models	CCR2^{+} (51)			CD206^{+} (52)		CD163^{+} (53)
AD Models	CX3CR1^{+}LY6C^{low} (54)			SR-B1 (55) CCR2 (56) CD36 (57)	TREM2^{+} (58)	CD45^{high} CD11b^{high} CCR2^{+} (59)

of immune and non-immune dwellers including leptomeningeal macrophages (lpMs). The SAS is contained between the tight arachnoid membrane and the pia mater, a thin monolayer of cells linked by desmosomes and gap junctions (104–107). Different collagen-rich trabeculae covered by pial/leptomeningeal cells connect the arachnoid to the pia mater in humans (103). Finally, below the pia mater, the glia limitans functionally separates the SAS from the parenchyma delineating the entire CNS (10, 13).

TABLE 2 | The table indicates the main RNA and protein markers described for macrophage populations in the distinct CNS compartments in human samples at steady state (homeostasis) and upon development of different CNS pathologies.

Human	Circulating Monocytes	BAMs (general markers)	lpMs	pvMs	cpMs	MdMs
Location →	**Blood**	**CNS borders**	**Leptomeninges**	**Perivascular Spaces**	**Choroid Plexus**	**CNS**
Homeostasis	$CD14^{high}$ $CD16^{-}$ $CD14^{+}$ $CD16^{high}$ $CD14^{high}$ $CD16^{+}$ (32, 60, 61)	*Stab1*, *Ch25h* (62)			$Iba1^{+}CD68^{+}MHCII^{+}$ (majority) $MHCII^{negative}$ $Iba1^{+}$ cells (minority) (63, 64)	
MS	$CD14^{+}$ $CD16^{high}$ (65) $CD14^{high}CD16^{high}$ (66)		Yolk Sac derived: CD68+ (67) CSF monocytes (bone marrow derived) *Cd9*, *Cd163*, *Egr1*, *Btg2*, *C1qa*, *C1qb*, *Maf*, *Csf1r*, *Stab1*, *Ch25h*, *Lyve1*, *Trem2*, *Tmem119*, *Gpr34* (62) $Cd16^{+}$ $CCR5^{high}$ $CD64^{+}$ $CD86^{+}CD14^{high}$ (68) HLA-G (69) $HLA\text{-}DR^{+}$ $CD33^{+}$ $Lyve1^{+}$ (70) $CD14+$ $FCGR3A/CD16^{intermediate}$ (62)	CD68, CD64, CD40, CD32, MHCII CD163, CD206 (71, 72)	$Iba1^{+}CD68^{+}MHCII^{+}$ (majority) $MHCII^{negative}$ $Iba1^{+}$ cells (minority) (63, 64)	Pv MdMs: *Nrf2* (73)
TBI				$CD163^{+}$ (74)		$CD14^{+}$ (75)
PD			CSF monocytes: $MHC\text{-}II^{+}$ (76)	$CD206^{+}$ (52)		$CD163^{+}$ (77) $CCR2^{+}$ (78, 79)
AD					$TREM2^{+}$ (80)	$CD163^{+}$ (77)

Importantly, the CSF permeating the SAS also fills the perivascular spaces of parenchymal vessels, with complex exchanges at the level of penetrating arteries surrounded by a layer of pial cells (108). The CSF also collects antigen-rich interstitial fluid from the CNS parenchyma (106), although the extent of this process remains the subject of debate. Accordingly, intra-CNS administration of drugs or tracers [e.g., intra-ventricular injection of clodronate particles (109)] leads to targeting of both lpMs and pvMs (88, 95), an often-overlooked phenomenon in BAM literature.

Altogether, both pvMs and lpMs continuously surveil CSF composition and thus indirectly examine the CNS at a molecular level (12). Given the high local production of immune-regulatory molecules such as TGFβ2 and IL13, the CSF can also influence the phenotype of resident SAS cells (110).

Long-lived lpMs originate in the yolk sac and seed the SAS embrionically (14). Similarly to pvMs, lpMs show an impaired potential for self-renewal following drug-induced inhibition of Csf1r, at least compared to fast-proliferating microglia (16).

LpMs constitute approximately 1/3 of the cells collected from human CSF (111) but are also found in high densities in the subpial layer above the parenchyma (38). Within the SAS, they are often located nearby fibroblast-like leptomeningeal cells (14). Morphologically, lpMs have been described as sessile elongated cells following leptomeningeal vessels (100). Recent intravital observations showed, however, that lpMs are heterogenously able to remain stationary with continuous ameboid movement or to crawl within the SAS (14).

As other BAMs, lpMs are $CD163^{high}CD206^{high}$ sentinels for pathogens and inflammation (33, 34, 49) and important sources of the chemoattractant CXCL12/SDF-1, a key factor in the migration of immune cells and neuronal and oligodendrocyte precursors (112, 113). On a transcriptional level, homeostatic lpMs express high levels of *Pf4*, *Cbr2*, *Ms4a7*, *Stab1*, *Fcrls*, and *Siglec1*, with certain subpopulations expressing *Cxcl2* and *Nfkbiz* (27). A different scRNA-seq study also indicated high expression of *P2rx7*, *Egfl7*, *Clec4n*, *Clec10a*, *Folr2*, and *Lyve1*, with a comparable expression pattern from birth to adulthood (16). Among these, Lyve1, a hyaluronic acid receptor highly expressed in lymphatic vessels (114), has emerged as a marker for $MHCII^{low}$ lpMs close to the pia mater (15), as opposed to its low expression in $MHCII^{high}$ lpMs in the SAS (115).

Interestingly, the SAS hosts a small population of $CX3CR1^{low}Lyve1^{+}CD38^{+}$ lpMs (15, 16) which might have escaped characterization in studies discriminating BAMs based on CX3CR1 positivity (14, 27).

Resident Dural Macrophages

The dura mater is the outermost component of the meninges, containing a high density of collagen and blood vessels that lack a BBB (104, 116). Differentiating this compartment from the rest of the CNS and similar to peripheral organs, the dura displays lymphatics running along major venous sinuses (93) and thus cannot be considered a CNS immune barrier (13, 28). Furthermore, the dura remains delineated from the SAS by a functional BCSFB containing intercellular tight junctions, the

impermeable arachnoid membrane (13, 104, 116). While the possible transit of immune cells from the dura to the CNS parenchyma remains unclear, different interchanges between dura and SAS can occur and remain an active area of study (97). Recent investigations have highlighted direct venous connections allowing neutrophils and potentially other myeloid cells to transit between the brain dural vasculature and the skull bone marrow (117, 118).

Dural resident macrophages are characterized as a dense Lyve1^{low}MHCII^{high} population, with few Lyve1^{high}MHCII^{+} cells present (15) in a different relative proportion compared to the SAS (119). These cells dynamically surveil the local environment while sensing distal gut biome changes (16). Displaying a mixed embryonic and bone marrow origin (6), dural macrophages account for the vast majority of blood-derived myeloid elements found in CNS preparations during homeostasis, together with CCR2^{+} macrophages within the ChP stroma (26). During inflammation, further blood-borne monocytes are locally recruited (97), while dural macrophages can regulate lymphoangiogenesis through the release of VEGF-C (120).

Resident Choroid Plexus Macrophages

ChPs are located within the third, fourth, and lateral ventricles of the brain and host a functional barrier for immune cell trafficking, the BCSFB. Separating peripheral circulation from the CSF, this barrier consists of a monolayer of epithelial cells connected through tight and adherens junctions (13) and expressing regulatory factors such as macrophage migration inhibitor factor (MIF) (121). On the basolateral side of this layer, a basement membrane and a thin stroma divide the BCSFB from fenestrated blood vessels (122, 123).

Producing the CSF and maintaining its chemical balance, the ChP has been considered as "the kidney of the CNS", indispensable for homeostatic equilibrium (124–126). Furthermore, the ChP plays roles in brain development, neurogenesis, metabolism (108, 127, 128) and secretes immunomodulatory microRNAs (129). The CSF itself has mechanical and signaling roles exerted through bioactive molecules and physical/chemical properties such as pH, osmolarity, and flow speed (130).

Different macrophages populate the ChP, albeit at a lower density compared to other CNS interfaces (26). ChP macrophages have been historically described as stromal phagolysosome-rich CD163^{+}MHCII^{+} antigen presenters (38, 39). Recent studies, however, indicate that the ChP hosts a highly heterogeneous population of yolk sac-derived long-lived stromal macrophages (ChPMs), CCR2^{+} blood-borne macrophages, and Sall1^{+} Kolmer/epiplexus cells situated on the apical side of epithelial cells and thus beyond the BCSFB (16).

The dynamic movement of ChP macrophages has been recently described by *in vivo* two-photon imaging following deep-brain cannula implantation: while epiplexus cells display different kinetic patterns on the apical side of epithelial cells, stromal macrophages continuously surveil ChP vasculature with highly motile processes, efficiently phagocytosing blood-borne fluorescent dextran (131).

Unique among BAMs, epiplexus macrophages share ontogeny, local self-renewal upon depletion, and transcriptome with parenchymal microglia (16). Analysis of the ChP *via* scRNA-seq identified three macrophage clusters sharing high expression of BAM signature genes *Mrc1*, *Ms4a7*, *Pf4*, *Stab1*, *Cbr2*, and *Fcrls* (27). Another scRNA-seq study also described three ChP clusters sharing signature expression of *Lilra5* and *Ttr* and identified as *Cst7*$^{+}$*Gm1673*$^{+}$ *Clec7a*$^{+}$ epiplexus cells, MHCII^{high} and MHCII^{low} ChPMs, the latter two likely corresponding to yolk sac- and bone marrow-derived resident ChPMs, respectively (16). In parallel, mass cytometry indicates equal numbers of Lyve1^{+}MHCII ^{negative}, Lyve1^{negative}MHCII^{+}, and Lyve1^{+}MHCII^{+} ChP macrophages, in a proportion which differs from the one observed at other CNS barriers (15).

Interestingly, MHCII expression in ChP macrophages is affected by microbiome alterations likely sensed *via* proximal fenestrated capillaries (16). Unfortunately, the effect of gut flora alterations has not been convincingly investigated in other BAMs.

CIRCULATING MONOCYTES, BORDER TRESPASSERS UPON INFLAMMATION

Origin, function and classification of blood monocytes have been reviewed elsewhere (32, 132–134). Briefly, following monopoiesis, monocytes are mobilized by a CCL2-dependent mechanism from the bone marrow and from splenic secondary reservoir (135) and enter the circulation displaying a half-life of approximately 1–2 days in mice and of 1–7 days in humans, depending on the cellular subset (22, 136–138). In the mouse, two major types of blood monocytes can be described as Ly6C^{high}CCR2^{+}CX3CR1^{low} "classical" inflammatory monocytes and Ly6C^{low}CCR2^{low}CX3CR1^{high} "non-classical" patrolling cells (31), with the latter originating from the former both in lymphoid organs and in the periphery (133). While Ly6C^{high}CCR2^{+}CX3CR1^{low} cells show fast CCR2-mediated recruitment toward inflamed tissues (139), patrolling monocytes mostly participate in endothelial homeostasis within the lumen (137, 140, 141). In humans, a parallel classification exists with "classical" monocytes characterized as CD14^{high}CD16^{negative}, non-classical cells as CD14^{+}CD16^{high} and transitional intermediate monocytes as CD14^{high}CD16^{+} (32, 60, 61). A more complex categorization of monocyte subtypes is, however, possible and advisable both for mice and human studies (142–145).

Despite their population-specific differences, all circulating monocytes express high levels of Csf1R and the receptor for granulocyte-monocyte colony stimulating factor (GM-CSFR), platelet endothelial cell adhesion molecule 1 (PECAM-1), and β2 and αM integrins, among others (32, 132).

Monocytes sense inflammation and damage *via* cytokines, chemoattractants, and damage-associated molecular patterns (DAMPs) which contribute to their tissue recruitment (146), with extravasation leading to differentiation to monocyte-derived macrophages (MdMs) (147). Depending on the specific context and highlighting their plastic potential, monocytes can, however, also differentiate into monocyte-derived DCs (148–150) or even to other cellular fates (151).

Dynamic interaction with endothelial cells in the vascular lumen involves a selectin-dependent rolling, a chemokine-

dependent arrest and adhesion, and an integrin-mediated crawling eventually resulting into diapedesis (152). Extracellular matrix molecules such as heparane sulfate proteoglycans expressed by the CNS vasculature can also mediate monocyte interaction with endothelial cells (153). Given a differential expression of interaction molecules and chemokine receptors, monocyte subtypes display intrinsic variance in this multistep process (132). Cell deformability through cytoskeletal reorganization and membrane stiffness changes are also regulators of trafficking (154). During trans-endothelial migration, monocytes interact with the endothelial molecules CD99, PECAM1 and CD155 (155) and, following diapedesis, cross the vascular basement membrane and interact with other perivascular cells (144, 156).

Within inflamed tissues, MdMs display substantial differences compared to monocytes. Upregulation of cell differentiation and trafficking genes starts during the first luminal contact with endothelial cells (144, 157, 158), with transmigrated monocytes showing significant changes in metabolism, chemotaxis, survival, inflammatory response (159), and rearrangement in subcellular structures leading to an augmented size (134). Altogether, through the recruitment process, monocytes can acquire distinct pro- or anti-inflammatory polarizations, substantially contributing to pathogen eradication/tissue destruction or to the regulation of inflammation/promotion of tissue regeneration, respectively.

MACROPHAGE PRO- AND ANTI-INFLAMMATORY FUNCTIONS

The acquisition of a functional phenotype by tissue macrophages and MdMs is a highly dynamic process which integrates several local cues and thus remains challenging to define *in vivo*. While these functional adaptations can be modeled and described in high detail *in vitro* (160) through a variety of techniques (161), the signaling pathways and functional activations observed *in vitro* and *in vivo* may diverge significantly depending on the model and the context (162).

Macrophage gene expression displays an inherent plasticity influenced by local signaling, chemical changes and physical confinement (163, 164). While pro-inflammatory macrophages mainly contribute to damage and neurotoxicity by the secretion of chemokines, inflammatory cytokines, and reactive oxygen and nitrogen species, anti-inflammatory cells extensively contribute to neuroprotection by debris scavenging and by releasing tissue regeneration intermediates and growth factors. Functional specifications are also reflected by divergent metabolic adaptations, with pro- and anti-inflammatory polarizations distinctively characterized by differential ATP production and oxygen consumption rates (165). Notably, the acquisition of a specific macrophage phenotype varies substantially also between different mouse strains (166).

To describe the spectrum of macrophage functions, researchers have largely made use of the M1/M2 dichotomy, a jargon introduced in the 1990s to indicate the outcomes of cellular stimulation with IL-4 or lipopolysaccharide (LPS)/IFNγ, respectively (167). Unfortunately, the application of the binary M1/M2 nomenclature to extremely diverse *in vitro* and *in vivo* contexts was unable to properly define multifaceted cellular actions (162, 163, 168). The limitations of this dichotomy were also evidenced when studying microglia/macrophage activation in several pathological contexts, including traumatic and neurodegenerative diseases and disease models (165). While some efforts of clarification in macrophage nomenclature have been made (25, 168–170), a generally accepted consensus is still missing. As suggested by experts in the field (170), we support a jargon describing cellular phenotypes *via* the *in vitro* stimuli used or, in complex *in vivo* scenarios, *via* the observed pro- or anti-inflammatory roles of the described populations.

Besides nomenclature issues, however, differentially polarized macrophage and monocyte subsets from mice and humans possess distinct migratory properties, for example, toward plasminogen (171). Notably, their CNS-invading trajectories and the anatomical site in which they acquire their differential function remain an undeveloped area of study.

MONOCYTE TRAFFICKING THROUGH THE CNS AT STEADY STATE

While accumulation of peripheral immune cells at CNS borders is a hallmark of CNS diseases (172), rapid recruitment of monocytes to perivascular CNS spaces is also observed upon peripheral inflammation, such as in endotoxemia (173). While this highlights the potential for active CNS surveillance by blood-borne myeloid cells notwithstanding the absence of local damage, CNS interfaces at steady state host only a limited number of bone marrow-derived immune cells (89, 174, 175). Recruitment of these cells drastically depends on local tissue accessibility, with interfaces such as the dura mater and the ChP hosting fenestrated vessels and a concomitant higher density of monocytes (16).

Importantly, stromal accumulation of blood-borne leukocytes in the ChP might serve as an intermediate step for reaching the CSF by crossing the BCSFB (176, 177). Analysis of human CSF indicates that approximately 1/3 of the cellular compartment comprises monocytes (178), with a vast majority of blood-borne $CD16^{high}$ cells (68). The homeostatic recruitment of these cells, potentially extravasating at the ChP or directly through leptomeningeal vessels as shown upon CNS damage (47, 112), is, however, unknown.

Given the secluded intraventricular location of ChPs, *in vitro* models have contributed significantly to our understanding of local cell trafficking (179). Using primary ChP mouse epithelial cells, we recently showed that MdMs can migrate through the BCSFB epithelium also in absence of inflammation (37). This transmigration pathway seems possible also for other myeloid cells (180).

Within the CNS parenchyma, basal immunesurveillance is exerted by microglia and pvMs, without apparent contributions by MdMs (14). The ability of MdMs to surveil these border areas at steady state has been historically overestimated due to the absence of tools discriminating yolk sac- and bone marrow-

derived myeloid cells and due to the experimental use of chemotherapy or gamma irradiation, artificially increasing BBB permeability and CNS chemokine production (15, 16, 22).

In general, the concept of peripheral immunesurveillance implies that patrolling antigen-presenting cells scan their target organ and, upon infection, move toward secondary lymphoid organs to trigger antigen-specific lymphocyte activation. Key to its relative immune privilege, however, the CNS shows limited afferent routes for cell-mediated antigen drainage (1). Antigen-rich CSF drains to peripheral venous blood *via* arachnoid villi and granulations and to the lymphatic system along nerve roots and nasal and dura lymphatics (12, 181). Notably, the relative importance of these pathways is still under debate (181, 182). Through these exit routes, CNS antigens can accumulate in peripheral lymph nodes (182, 183), potentially *via* DCs trafficking from CNS borders to peripheral organs (28, 184). Whether monocytes and MdMs can also participate in this afferent arm of CNS immunity in a comparable manner to that observed in peripheral tissues (185) is, however, unclear (175).

MYELOID DWELLERS AND TRESPASSERS AT CNS INTERFACES UPON AUTO-AGGRESSIVE CNS INFLAMMATION

Macrophages constitute the predominant cell type in the damaged CNS of multiple sclerosis (MS) patients, independently from clinical course (169) and lesion subtype (186, 187). Accordingly, MS disease-modifying therapies strongly affect monocyte/macrophage functions as part of their therapeutic action (169, 188–190).

MS is a chronic inflammatory disease of the CNS with unknown etiology and a heterogeneous pathological course, including relapsing-remitting (RRMS), primary and secondary progressive forms (191). Histopathologically, MS is characterized by multifocal BBB damage and leukocyte infiltration in lesions displaying demyelination and neuronal death (192, 193). To date, whether neurodegeneration is the primary cause or rather the secondary consequence of auto-aggressive inflammation remains debated (194).

Blood monocytes isolated from MS patients show altered expression of microRNAs (195), microvescicle release (196), cytokines (197), norepinephrine (198), and enhanced CCL2-, CCL5-, and CXCL1-driven migration (188, 199) compared to cells from healthy controls. The relative proportions of circulating classical, intermediate, and nonclassical monocytes varies across studies, with some indicating a substantial increase in nonclassical $CD14^{+}CD16^{high}$ monocytes (65), while recent work shows an increase in $CD14^{high}$ and $CD16^{high}$ monocytes specifically in RRMS patients with inactive disease (66).

Within the CNS parenchyma, resident and invading macrophages play complex roles both preclinically and in established lesions (71). Monocyte invasion might, however, vary at different disease stages, with less MdM infiltrates observed in progressive MS compared to RRMS (200).

Inflammatory macrophage functions range from tissue destruction (103) to beneficial roles (201, 202), a continuum reflecting their unique transcriptional plasticity (163, 170). While microglia actions during MS fall in the same context-dependent classification, slowly expanding lesions from progressive MS patients display high density of pro-inflammatory markers in perilesional microglia, showing how these cells can contribute to disease progression (203). In general, however, it remains unclear whether distinct microglia/macrophage actions are preferentially associated with different phases of lesion evolution, or whether they co-exist at every clinical timepoint or even within the same cells (72, 169).

Albeit heterogeneous, the distribution of MS lesions often follows an expected pattern (204), potentially shaped by routes of leukocyte entry and local antigen presentation (205).

To mimic the multifaceted pathological aspects of MS, several inducible and spontaneous animal models have been established. Among these, experimental autoimmune encephalomyelitis (EAE) has been the main tool to study disease mechanisms and to develop and test MS disease-modifying therapies (169), despite its intrinsic limitations as an MS model (206).

As in MS, inflammation in EAE is characterized by a high density of activated macrophages at CNS interfaces and within parenchymal lesions (**Figure 2**) (44). Given the overlapping expression of key markers including CD11b, Csf1R, CD163, and CD206 (21), the relative pathological contributions of MdMs and resident macrophages has remained unaddressed for decades, but technical advancements finally allow us to define their respective roles (169). Upon induction of EAE, BAMs increase their expression of MHCII, CD44, the immunomodulatory molecule PDL1, CD117 (c-KIT), and Sca-1 (Ly6a) (15). Despite convergent morphological and expression changes, resident macrophages and MdMs remain transcriptionally separate (208) and can be distinguished through mass cytometry (15) and scRNAseq techniques (27). The survival dynamics of recruited MdMs remain, however, unclear, with previous work indicating an inability of invading macrophages to persist as microglia-like cells (209) and recent reports showing the opposite (21, 210, 211).

During the active phase of EAE, MdMs substantially outnumber BAMs at CNS interfaces (15). Compared to circulating monocytes, CNS-recruited MdMs upregulate glycolytic enzymes and production of inflammatory molecules (44). While experimental MdM removal by apoptosis reduces disease burden (212), MdM accumulation directly correlates with neurodegeneration (208), likely through an increased production of reactive nitrogen and oxygen species (ROS/RNS) (213, 214). Altogether, monocyte infiltration into the CNS parenchyma parallels EAE clinical signs and exerts a significant influence on glial cells (215, 216), at the same time contributing to tissue regeneration (217, 218).

The overall complexity of macrophage phenotypes during EAE is exemplified by the differential expression of the enzymes iNOS and arginase-1 in $CCR2^{+}Ly6C^{high}$ MdMs (44). While $iNOS^{+}$ MdMs revealed higher glycolytic rates, expression of matrix metalloproteinases (MMPs), chemokines (e.g., *Ccl5*), and pro-inflammatory cytokines, $arginase\text{-}1^{+}$ MdMs showed increased expression of scavenging receptors, complement

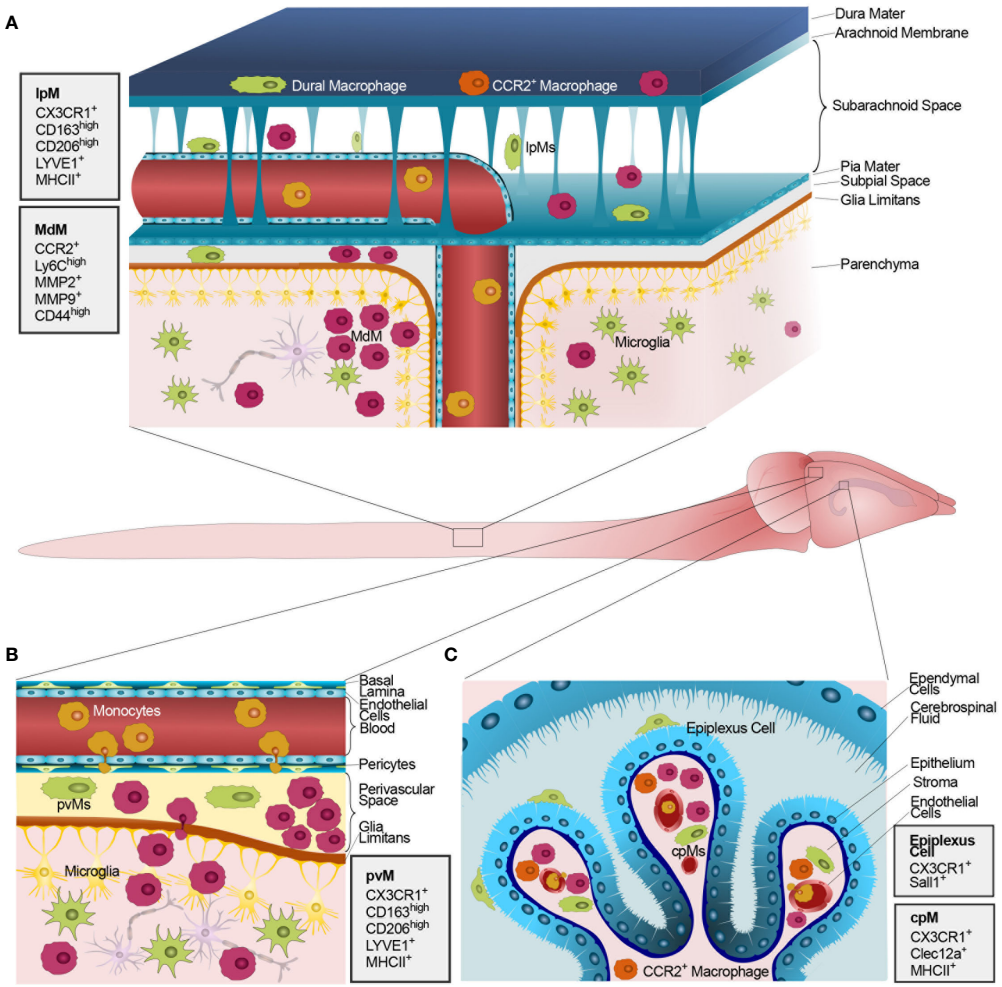

FIGURE 2 | Macrophages populating CNS barriers and parenchyma during autoimmune CNS inflammation. The figure shows the mouse CNS following induction of EAE and disease development. The magnified inlets illustrate schematic representations of the anatomical CNS interfaces containing functional barriers and blood-borne MdMs. **(A)** The mouse meninges. Top to bottom, the dura mater hosts yolk sac-derived (green), blood-borne CCR2$^+$ macrophages (orange), and several activated MdMs (red). Different monocytes/MdMs (red) infiltrate the SAS and the subpial space either extravasating at the level of leptomeningeal vessels or crossing the distal ChP BCSFB, thus reaching the CSF. MdMs can invade the CNS parenchyma while yolk sac-derived microglia (green) increase in number. **(B)** Schematic representation of the perivascular space at the level of post-capillary CNS venules, hosting yolk sac-derived pvMs (green) and invading MdMs (red) collectively forming a perivascular inflammatory cuff. After crossing the glia limitans, MdMs (red) accumulate in parenchymal lesions. **(C)** Schematic representation of the ChP within a CSF-filled brain ventricle lined by ependymal cells. The ChP stroma is enlarged compared to steady state and hosts a combination of yolk sac-derived cpMs (green) and different populations of blood-borne inflammatory CCR2$^+$ macrophages (orange/red) increasingly extravasated from stromal vessels lacking a BBB. Monocytes circulating within vascular lumens are shown in yellow. The epithelial cells of the BCSFB show decreased density of apical microvilli and are represented as darker and lighter cells to model the ultrastructural alterations previously reported (207). Next to each panel, gray boxes illustrate the main protein markers identifying CNS-resident macrophages in the distinct anatomical compartments, and the main markers commonly expressed by MdMs during CNS inflammation.

proteins and oxidative phosphorylation enzymes (44). Notably and beside iNOS$^+$ and arginase-1$^+$ cells, a recent study described two Saa3$^+$ and Cxcl10$^+$ monocyte populations substantially contributing to tissue damage within the CNS parenchyma (43).

Before reaching the white or gray matter, MdMs are increasingly recruited to the ChP stroma (37) and need to cross CNS barriers as shown by accumulation within perivascular cuffs at the BBB (45, 46) or in the CSF, extravasating from the ChP and/or from leptomeningeal vessels. Notably, massive monocyte/macrophage accumulation at CNS borders does not directly lead to clinical symptoms in absence of parenchymal infiltration (219). Passage through these interfaces allows, however, monocytes to adapt to the border microenvironment, acquire distinct functional polarizations and, in turn, regulate the evolution of the disease. Hence, the description of MdM migratory routes and the definition of the parallel role of BAMs represent a fundamental milestone in our understanding of auto-aggressive CNS inflammation.

Macrophages at the BBB During MS and EAE

BAMs efficiently sense the peripheral environment *via* bidirectional communication with their milieu and in particular with endothelial

cells (220), scanning for potential distal danger and reacting to it promptly (100, 221). Experimental evidences from both MS and EAE indeed suggest that pvMs become activated even before development of clinical symptoms and infiltration by peripheral cells (222, 223). Accordingly, pre-clinical CNS lesions observed in marmoset EAE models correlate with increased Iba1$^+$ pvMs (224). CNS inflammation leads to a sudden increase in pvM density and to augmented antigen-presenting capabilities (225). These pvMs also increase in number in EAE (42, 226) *via* a local proliferation lasting until the chronic disease phase (14). The observed proliferation rate is, however, minor compared to the one described for microglia (27).

Nearby MS lesions, pvMs express CD68, CD64, CD40, CD32, and MHCII, as well as the signature proteins CD163 and CD206 (71, 72). During their activation, pvMs also upregulate expression of interacting molecules such as ICAM-1 and VCAM-1 and chemoattractants such as CCL2/MCP-1 and CCL3/MIP-1α (42). Notably, in both EAE and MS, pvMs appear highly phagocytic and take up substantial amounts of iron, directly linked to demyelination (227, 228).

Surprisingly, however, scRNA-seq analysis indicates that pvMs undergo only mild modifications to their overall transcriptional profile during EAE, compared to their steady state (27). Among the few significantly regulated genes, an increased expression of *Ccl5*, a T cell chemoattractant, of the MIF receptor *Cd74* (41) and a decreased expression of *Lyve1* and *Ctsd* (Cathepsin D, a bactericidal protein) was observed (27).

In parallel, neuroinflammation correlates with massive recruitment of peripheral monocytes which cross the BBB drastically increasing the cellularity of perivascular spaces. Monocyte mobilization from peripheral reservoirs is regulated by several factors including GM-CSF (149), a cytokine playing key roles in both EAE and MS (229).

MdM recruitment results in the formation of perivascular cuffs, a MS pathological hallmark where lymphocytes intersperse with a majority of CD16^{high} myelin-laden MdMs (208, 230). At least in EAE, perivascular MdMs appear morphologically smaller than activated resident pvMs (27). A series of DAMPs/alarmins including HGMB1, IL33, and ATP participate in the recruitment of monocytes (146). In turn, both *in vitro* (68) and *in vivo* data (45) indicate that MdM perivascular accumulation enhance recruitment and parenchymal invasion of lymphocytes.

Perivascular inflammatory cell cuff formation is often associated with BBB disruption, a multifaceted concept entailing exogenous and endogenous mechanisms (231). Even though BBB damage aid monocyte extravasation, immune cells can cross endothelial barriers showing intact intercellular tight junctions (3, 37, 190). Monocyte diapedesis is aided by the release of inflammatory molecules such as tissue transglutaminase 2, oncostatin M, histamine, superoxide, GM-CSF, and TNFα (232–235). Contact with endothelial cells involves interactions between monocyte integrins such as VLA-4/LFA-1 and endothelial integrin-binding molecules such as VCAM-1/ICAM-1 (169), homophilic interactions through Ninjurin1 (236) and expression of the constitutively expressed adhesion molecule CD166 (237). Mechanistically, contact of monocytes with the BBB allows release of tissue plasminogen activator from endothelial cells following activation of the NMDA receptor NR1, allowing, in turn, monocyte diapedesis (238, 239). Perivascular cell cuff formation is also dependent on the local expression and accumulation of chemokines, including CCL2 (240), CCL3, CXCL12 (46), and potentially of the constitutively expressed CCL19 (241).

In particular, CCL2 actions have been extensively studied in MS and EAE (242). This chemokine can exert distinct roles depending on its astrocytic or endothelial source (240). CCL2 regulates CCR2$^+$ monocyte adherence and chemotaxis by acting on monocyte integrin conformation and clustering (243–246). Following tissue invasion, CCR2 expression is downregulated contributing to CNS retention of inflammatory MdMs as observed *in vivo* (44) and *in vitro* (37). Signaling through CCR1 and CCR5 can similarly regulate monocyte multistep accumulation in the perivascular spaces, collectively guiding disease development (185, 247).

Despite its intrinsic pathological consequences, the accumulation of MdMs in perivascular cuffs also constitutes an efficient checkpoint mechanism ensuring that cells do not indiscriminately enter in contact with neuronal cells. To infiltrate the CNS parenchyma from the perivascular space, immune cells need to additionally cross the parenchymal basement membrane and the glia limitans (45). Notably, during neuroinflammation, BBB disruption induces expression of tight junctions in astrocytic end-feet in both EAE (248) and MS (249). Crossing of this second barrier crucially requires expression of MMPs and of MMP regulatory proteins such as CD147 (169). In this process, MMP2 and MMP9 participate in the lysis of perivascular chemokines such as CXCL12, that increase retention of MdMs preventing parenchymal infiltration (45, 46).

Are perivascular MdMs functionally polarized during anti-CNS responses? While interacting with endothelial cells, transmigrating monocytes encounter several activation signals. GM-CSF, shown to augment monocyte diapedesis at the BBB, can induce a hybrid inflammatory phenotype similar to the one observed in MS tissues (232). GM-CSF can also be released by endothelial cells (250, 251) upon stimulation by Il-1β, an inflammatory cytokine released by invading monocytes and indispensable for their infiltration (251), in a looping mechanism potentiating MdM activation between BBB and parenchyma (252). Furthermore, feedback regulation by reactive species-mediated quorum-sensing might play a key role in phenotype adaptation (30). MdMs within the perivascular space can have an iNOS$^+$ or Arginase-1$^+$ phenotype; furthermore, *in vivo* imaging indicated that MdMs acquired a pro-inflammatory state immediately following diapedesis and before entering the CNS parenchyma (44). Accordingly, perivascular accumulation of glycolytic phagocytes has been observed in both EAE and MS, with recruited MdMs reducing their rate of glycolysis once in the parenchyma (253). Inhibition of glycolysis and of lactate secretion reduced macrophage invasion, both *in vivo* and *in vitro* (253). MdMs in perivascular cuffs were strongly positive for *Nrf2*, a transcription factor involved in protection against oxidative stress and highly expressed in acute MS lesions (73). Moreover, in both MS and EAE, these cells upregulate expression of the extracellular matrix components lectican versican V1 and chondroitin sulfate

glycosaminoglycans, molecules able to enhance MdM migration and secretion of inflammatory cytokine and chemokines (254). Finally, pvMs in active demyelinating MS lesions also show strong reactivity for TGFβ (255), an anti-inflammatory molecule with controversial roles (256).

Taken together, the perivascular space thus appears like a key compartment able to shape the pathological role of recruited monocytes in their migration toward the inflamed parenchyma.

Macrophages in the SAS During MS and EAE

Meningeal inflammation is common in MS clinical manifestations, including primary progressive forms (67, 257), often correlating with neurodegeneration (258). The meninges of MS patients can also host lymphoid follicle-like structures rich in B lymphocytes, potential drivers of disease (259).

However, histological analysis reveals that $CD68^+$ myelin-laden phagocytes are the most represented cell type in the inflamed SAS (67). Collection of CSF *via* lumbar puncture allows analysis of non-adherent SAS phagocytes and indicates significant variations in CSF cellularity depending on the MS subtype. Compared to healthy donors, the CSF of RRMS patients shows a relative decrease in $CD16^+$ monocytes and an increasing proportion of $CCR5^{high}CD64^+CD86^+CD14^+$ monocytic cells (68). The described decrease in $CD16^+$ monocytes is not observed in primary progressive patients, potentially reflecting different recruitment mechanisms (68). Other studies have shown an increased presence of monocytes expressing HLA-G, an immunosuppressive non-classical MHC molecule (69). Notably, a recent scRNA-seq analysis of the CSF of MS patients identified a majority of monocytes expressing HLA-DR and the lectin CD33 and a small population of $HLA\text{-}DR^{high}CD33^{mid}Lyve1^+$ macrophages identified by the authors as "microglia" due to their expression of *Trem2* and *Olr1* (70). This jargon is, however, misleading, as no evidence of parenchymal microglia crossing the glia limitans toward the CSF exists. In another study, a comparable population (named "Mono2") showed expression of BAM markers such as *Stab1* and *Ch25h* and of inflammatory genes such as *Cd9*, *Cd163*, *Egr1*, *Btg2*, *C1qa*, *C1qb*, *Maf*, and *Csf1R* (62).

Notwithstanding their controversial classification, SAS lpMs clearly play a key role in MS/EAE by producing inflammatory molecules such as CCL5, CXCL9, CXCL10, and CXCL11, in turn, recruiting further leukocytes into the CSF (112, 260).

In EAE, lpMs increase their Iba1 immune reactivity several days before clinical onset of disease, thus indicating a role in the initiation of local inflammation (40). LpMs are highly dynamic dwellers and interact extensively with invading T cells, increasing their proliferation rate during the acute EAE phase (27, 112). This increase in number drops during the chronic phase of disease, paralleled by local appearance of apoptotic lpMs (27). Notably, similar to pvMs, lpM activation in EAE does not lead to dramatic transcriptome changes compared to homeostatic conditions (27).

In MS and EAE, monocytes/MdMs also accumulate in large numbers in the SAS following extravasation from the leptomeningeal vasculature or from the ChP and CSF-filled ventricles. Infiltration through leptomeningeal vessels follows increased intraluminal monocyte crawling and expression of the enzyme tissue transglutaminase 2 (261), known for its involvement in cell adhesion to fibronectin, a glycoprotein released by endothelial cells and pericytes (262).

MdM and lpM dynamics in the SAS during EAE have been extensively explored by intravital imaging (223). Preclinically, lpMs cluster around leptomeningeal vessels following leakage of plasma fibrinogen, which, in turn, triggers ROS production (222). Studies in rats have demonstrated that meningeal phagocytes can present both self and non-self antigens and thus activate infiltrating T cells in a multistep process requiring chemokine signaling (112, 263–265).

Long-lasting contacts seem to occur preferentially between lymphocytes and blood-borne $CCR2^+$ monocyte-derived cells, rather than with resident BAMs (27).

Surprisingly, however, meningeal macrophages do to play an essential role in antigen presentation during EAE. A series of recent reports convincingly demonstrated that expression of MHCII in $CD11c^+$ classic DCs but not in $CX3CR1^+$ macrophages is indispensable for disease induction (28, 266, 267). Nonetheless, the frequency of lpM and MdM contacting lymphocytes suggests the existence of further regulatory roles shaping EAE. In our work, the majority of SAS MdM displayed strong expression of arginase-1^+ in striking contrast to the $iNOS^+$ dominated nature of parenchymal lesions, potentially indicating an anti-inflammatory function of lpMs (44). The differential representation of MdM phenotypes in the SAS and in other CNS compartments might also be related to distinct sensitivity toward chemoattractants, as shown *in vitro* using differentially polarized human cells (268, 269), either as a result of differential chemokine receptor expression or a differential receptor response to transduction.

From the SAS, activated MdMs can reach the CNS parenchyma and participate in the formation of subpial demyelinating lesions, an histological hallmark of progressive MS forms (191). The contribution of MdMs, however, depends on the type of lesion, with so-called leukocortical plaques showing a high number of activated macrophages and purely subpial cortical lesions mostly devoid of inflammatory infiltrates (257). In EAE, subpial white matter demyelination is commonly described in the spinal cord, but cortical gray matter pathology, as observed in MS, is rare. The latter type of lesion can, however, be modeled in mice through cortical injections of TNF and IFNγ (270, 271) or by peripheral injection of β-synuclein-specific T cells (272).

To reach the CNS parenchyma from the SAS, meningeal MdMs need to transverse the pia mater, the parenchyma-associated basement membrane and, eventually, the glia limitans (10). *In vivo* imaging has shown that cells within the SAS might move toward the parenchyma by crawling on the external surface of leptomeningeal vessels entering the parenchyma (273). However, the permeability of the pia mater to immune cell trafficking remains debated, and the required interaction molecules are unknown (104, 107, 260).

Migration of MdMs from the meninges to the parenchyma can be downregulated by the administration of CXCR7 antagonists, impeding CXCL12 signaling and resulting in

meningeal accumulation (274). Retention of phagocytes in the SAS was paralleled by loss of VCAM-1 on astrocytes, thus highlighting a potential role of these cells as interactive partners in the invasion process (275).

To summarize, even though anatomical differences between the meningeal system in rodents and humans impede a fully coherent discussion, several reports have evidenced the central part played by leptomeninges in initiation and evolution of autoimmune CNS inflammation. Nonetheless, many unsolved questions exist regarding macrophage functions and trafficking routes. A detailed anatomical description of these compartments and the creation of transgenic animals allowing visualization of defined meningeal layers (116) remain crucially needed for the progress of the field.

Macrophages in the ChP During MS and EAE

In the context of auto-aggressive CNS inflammation, the ChP has been proposed as the first CNS gateway for autoreactive lymphocytes prior to BBB disruption, subsequently triggering a secondary leukocyte CNS infiltration driving disease progression (40, 260, 276).

Rather than a sealed barrier, the BCSFB is considered an active yet highly regulated exchange surface (108) showing a differential expression of tight junctions compared to the BBB (123).

Immune cell trafficking at the BCSFB seems to be regulated by IFNγ-dependent activation in immune surveillance and repair (277). Both CCL20 and CX3CL1 are constitutively expressed at the ChP and might guide recruitment of $CCR6^+$ (278) and of $CX3CR1^+$ leukocytes, respectively (279). The BCSFB is highly sensitive to systemic inflammation. Thus, peripheral LPS administration leads to local TNF and IL1β secretion, upregulates CXCL1 and CCL2 (280, 281), and triggers release of destabilizing MMP8 and MMP9 (282) and impairment of tight junction barrier properties, an overall reaction suggesting higher trafficking of immune cells.

In the ChP of MS patients, the tight junction protein claudin-3 is downregulated compared to healthy controls (283). Reports of its role in EAE models are, however, controversial, with its deletion increasing numbers of CSF-infiltrated MdMs in one study (283) and to a lack of BCSFB impairment in a recent report (284).

Interestingly, interaction molecules such as ICAM-1 and VCAM-1 are specifically expressed on the apical side of the BCSFB epithelium, facing the CSF (285). During EAE, their increased expression and a *de novo* apical expression of MAdCAM-1 can be observed (207). Notably, while leukocytes crossing the BCSFB toward the CSF utilize ICAM-1 in the last steps of diapedesis (286), the apical location of these molecules seemingly indicates that leukocytes can also migrate backward from the CSF to the ChP stroma (286, 287).

As shown by 2-photon microscopy, ChP macrophages readily respond to peripheral LPS injections by moving toward nearby vessels, with focal ChP damage leading to spatial reorganization of epiplexus cells around the injury site. In both scenarios not all macrophages responded to the danger stimuli, again highlighting the heterogeneity of ChP dwellers (131).

Compared to steady state, induction of EAE leads to the appearance of disease-associated ChPMs (27). These activated cells show significantly increased expression of antigen presentation molecules, chemokines and cytokines such as *Il1b*, with one cluster strongly positive for MHCII genes and for *Ctss* (encoding for Cathepsin S), and the other showing high expression of the antimicrobial products *S100a9*, *S100a8*, and *Ngp* (27). The presence of $CCR2^+$ MdMs in the ChP appears substantially enriched throughout the disease, with a minor proportion of these cells locally expressing iNOS and/or arginase-1 (37).

ChP MdM populations also show high CD74 positivity and can be divided in three different cellular clusters composed of $Ly6C^{high}$ monocyte-like, $Cd209^+$ DC-like and $MertK^+$ macrophage-like cells (27).

Beside observations in EAE, not much is known about ChP macrophages in MS. Analysis of human ChP tissue revealed a high density of $CD68^+MHCII^+$ macrophages and a minor proportion of $MHCII^{negative}$ $Iba1^+$ cells, with these cells present within the stroma, intercalated between epithelial cells or lying on the apical side of epithelial cells (63, 64). However, the densities of these cells appeared comparable between progressive MS patients and healthy controls (64).

Do MdMs really access the CSF *via* the ChP during autoaggressive neuroinflammation? In non-autoimmune disease models, monocytes/macrophages were indeed shown to cross the BCSFB toward the CSF (47, 288, 289). Using *in vitro* BCSFB models, we could recently confirm that functionally polarized mouse macrophages can actively migrate through the BCSFB monolayer (37). Apparently migrating $MHCII^+CD68^+$ macrophages have also been described interspersed between epithelial cells in the ChP of MS patients (63), yet these cells might represent DC surveillants bridging across the BCSFB (290).

To summarize, while in MS the gateway function of the ChP remains unsupported by direct evidence, an active role of the BCSFB in MdM recruitment to the CNS is highly plausable and this CNS interface should become a focus of attention in neuroinflammatory research.

MONOCYTE/MACROPHAGES AT CNS INTERFACES IN TRAUMATIC CNS INJURY

Despite shielding by bones, meninges, and CSF, traumatic damage to the CNS parenchyma is a common pathological occurrence leading to neurodegeneration and to an innate immune response promoting further tissue damage (291, 292). Physical insults can occur to the brain (traumatic brain injury, TBI) or to the spinal cord (spinal cord injury, SCI), with these two compartments showing the evolution of distinct pathologies (293). Depending on their severity, mechanical injuries to the CNS result in local death, DAMP release, activation of BAMs and to different degrees of MdM infiltration (146). Interestingly, compared to brain lesions, physical damage to the spinal cord

generally results into a higher activation of CNS macrophages, stronger BBB damage, and denser MdM accumulation (293).

Spinal Cord Injury

Following SCI, perilesional microglia proliferate creating a protective "microglial scar" in concert with astrocytes (294). Communication between microglia and infiltrating MdMs influences their reciprocal polarization as well as lesion evolution (215). While removal of microglia in SCI models proved detrimental, the role of BAMs in SCI was not convincingly addressed. One report showed that pvMs and lpMs do not participate in the disease process, nor do they proliferate extensively in response to SCI (294). The seemingly minor role of BAMs in SCI evolution is supported by observations in demyelinating models: following intra-parenchymal injections of lysophosphatidylcholine, Lyve1$^+$ lpMs and pvMs do not penetrate into demyelinated spinal cord lesions (48). Nonetheless, more research is crucially needed to clarify BAM participation in SCI.

Upon injury, spinal cord endothelial cells upregulate the expression of VCAM-1 and ICAM-1 allowing first neutrophils and later monocytes to accumulate in the damaged region (295). Interestingly, these myeloid cells originate mostly from the spleen reservoir pool rather than from the bone marrow, a pattern likely related to the acuteness of the disease (135, 296). Similar to what is observed in EAE models, sensing of CCL2 and CXCL12 and production of MMP9 are required for monocyte migration from the BBB to the parenchyma upon SCI (297, 298). CCL2 and other chemokines released by glial cells such as astrocytes might also contribute to the acquisition of a functional phenotype by invading and local macrophages (299). Local TNF-α production increases MMP-9 expression highlighting a complex interplay of cytokines and proteases. Once in the parenchyma, MdMs follow a gradient of C5a molecules toward the lesion epicenter in a mechanism regulated by IRF8-purinergic receptor axis, all leading to enhanced tissue repair (300). Accordingly, MdMs in SCI have been described as beneficial (146). Conditional ablation of CD11c$^+$ phagocytes during the first week after SCI resulted in worsened clinical recovery, however, did not affect the pathology when induced 2 weeks following damage (301). The beneficial effect of MdMs was attributed to their expression of anti-inflammatory IL-10 at the lesion margin (301). Notably, a follow-up work showed that while Ly6C^{hi}CX3CR1^{lo}CCR2^{hi} monocytes infiltrated the parenchyma in a CCL2-dependent manner *via* leptomeningeal vessels proximal to the lesion, anti-inflammatory Ly6C^{lo}CX3CR1^{high}CCR2^{lo} cells entered the CNS trafficking through the BCSFB (47). In this study, monocyte migration at the ChP relied on VLA4/VCAM-1 interactions as well as on the expression of the adenosine-catalyzing enzyme CD73. Intriguingly, it has been postulated that these polarization differences could be related to the cellular constituents of the two barriers, epithelial cells for the BCSCF and endothelial cells for leptomeningeal vessels (47).

Nonetheless, the net contribution of MdMs to SCI appears time- and location-dependent (302–305). Indeed, one report described locally recruited MdMs as pro-inflammatory players surrounded by anti-inflammatory microglia distal to the injured area (306). Acquisition of an anti-inflammatory phenotype is also affected by the activation status of nearby astroglial cells (299). In another work, inhibition of monocyte infiltration (via splenectomy) accordingly resulted into an ameliorated clinical phenotype (307). Lastly, other studies have observed that MdMs in the lesioned parenchyma show co-expression of pro- and anti-inflammatory markers, once more highlighting the non-binary role of MdMs in CNS injury and indicating complex local functions upon recruitment (308, 309).

Traumatic Brain Injury

Similar to SCI, TBI shows a long-term pathological evolution involving excitotoxicity, cytokine release, ROS/RNS production, and infiltrating myeloid cells with neurotoxic as well as neuroprotective functions (310). Beside the extensively investigated role of microglia (50), an involvement of BAMs upon TBI has been suggested by both human and animal studies.

In TBI patients, CD163$^+$ microglia/macrophages are increased in both the lesion and perivascular spaces indicating a potential participation of CD163$^+$ pvMs to damage evolution (74). CD163$^+$ cells are also increased in a rat TBI model two days post TBI, however, mainly within the lesion (49). Importantly, most CD163$^+$ cells co-expressed heme oxygenase-1 (49), a key enzyme in heme catabolism (311) exerting anti-inflammatory effects (312, 313) likely promoting neuroprotection following TBI. Nonetheless, in these studies, the peripheral or resident nature of pvMs was not convincingly defined.

Blood-borne MdMs play instead a recognized and context-dependent role in TBI (314). Pro-inflammatory macrophages are recruited early in the lesioned area, with CCR2$^+$ MdMs following gradients of chemokines released from activated parenchymal cells (50). Notably, CCR2$^+$ monocytes seem to mediate local ROS/RNS production (315, 316) and might thus constitute important pharmacological targets. Indeed, intravenous injection of immunomodulatory nanoparticles reduced MdM recruitment by affecting monocyte survival and sequestration within the spleen (190), altogether leading to a strong reduction of lesion volume (317).

Conversely, other studies have provided evidence for a beneficial effect of monocyte recruitment after mild TBI, for instance by reducing meningeal vascular damage (318), a pathological hallmark associated to peripheral immune response (319). In the latter study, while "classical" debris-scavenging monocytes were located at the center of meningeal lesions, wound-healing "non-classical" monocytes were localized peri-lesionally and promoted meningeal angiogenesis *via* expression of MMP-2 (318).

How do MdMs access the CNS parenchyma following TBI? Analysis of patient tissues suggests that CD14$^+$ monocytes initially migrate toward the perilesional perivascular space within the first days following damage and then move toward the parenchyma (75). These MdMs can remain in the perilesional area for weeks (75). In a rat model of TBI, monocytes were instead shown to enter the lesioned CNS parenchyma by crossing SAS microvessels, subsequently accumulating near the injury site (320). Some MdMs appeared to move a short distance along perivascular spaces toward the brain parenchyma (320). This trafficking route utilized by MdMs (and neutrophils) requires cellular interactions that can

also be mediated by JAM-A, a junctional adhesion molecule also expressed by macrophages (321, 322).

A potential role of the ChP as a monocyte access gateway in TBI models was suggested by the rapid increase in CCL2 production by the ChP ipsilateral to the lesion. The resulting rise in CCL2 CSF concentration mirrors observations in the CSF of severe TBI patients (323). BCSFB epithelial cultures indicated that CCL2 is secreted across both the apical and basolateral side, a bidirectional production necessary for leukocyte migration at the BCSFB following TBI (288). Accordingly, the blocking of this signaling reduced $CCR2^+$ monocyte infiltration and lesion volume (324) and improved neurological recovery after TBI (315, 323, 325). Interestingly, lack of MdM infiltration correlated with increased astrocyte proliferation and reduced astroglial scar formation, thus suggesting a key role of juxtavascular astrocytes in the interaction with MdMs (326).

Taken together, evidence for a potential involvement of BAMs in traumatic CNS disorders remains sparse. Furthermore, experimental approaches allowing selective investigation of yolk sac-derived macrophages have not yet been utilized in this context. The parallel role of CNS-infiltrating monocytes has been more extensively described and appear extremely dependent on recruitment timing, damage extent and lesion location. A better definition of the role of MdMs would potentially aid the development of novel therapeutics for patients suffering from Sor TBI. and/or TBI.

MYELOID DWELLERS AND TRESPASSERS AT CNS INTERFACES IN NEURODEGENERATIVE DISEASES

Parkinson's Disease

Parkinson's disease (PD) is a progressive neurodegenerative disorder characterized by neuronal loss in the substantia nigra pars compacta (SNpc) and by chronic CNS inflammation in both patients and animal models (327, 328). Histopathologically, brain samples of PD patients show accumulation of Lewy bodies rich in the neuronal protein α-synuclein (329). Post-mortem and imaging analysis of PD patients revealed detrimental microglial activation (330, 331) accompanied by upregulation of iNOS as well as production of pro-inflammatory cytokines within the parenchyma (332–334). Strong to moderate microglial activation is also observable in the different animal models of PD (327, 335, 336).

However, the role of BAMs in disease progression has rarely been addressed. In a viral model of synucleinopathy, degeneration of neurons in the SNpc coincided with local $CD206^+$ pvM expansion, similar to what has been observed in post-mortem samples of PD patients (52). Depletion of pvMs and lpMs by clodronate liposomes resulted in a significant loss of dopaminergic neurons within the SNpc after two weeks, suggesting that BAMs exert a neuroprotective function in the pathology (52). Notably, clodronate administration increased vascular expression of VCAM-1, enhancing CNS accumulation of lymphocytes (52). Absence of pvMs led to aggravated spreading of misfolded α-synuclein (52), a pathological hallmark of PD (337). On the other hand, in a 6-hydroxydopamine-mediated PD model, no increase in rod-like $CD163^+$ pvMs was found in the striatum as opposed to the recruitment from the blood stream of $CD163^+$ "polygonal" cells (53). Notably, these parenchymal $CD163^+$ cells are also described in the brain of PD patients in association with Aβ deposition and a damaged BBB (77). BBB impairments are commonly observed in both patients and animal models (338).

Several studies have documented an augmented presence of inflammatory cytokines in the CSF of PD patients (338), the increased expression of MHCII in CSF monocytes (76), the presence of α-synuclein at the BCSFB (282, 339) and a potential beneficial role exerted by ChP epithelial cells in transplantation experiments (340). Beyond this sparse evidence of a role of the ChP and in general of BAMs however, the potential contribution of these cells to PD evolution remains unexplored.

Conversely, several studies addressed monocytes/MdMs functions in PD pathogenesis. The total number of blood monocytes is not affected in PD patients (78), but these cells appear less responsive to activation and more proliferative (341) and show altered phagocytosis (78, 342). Furthermore, monocytes from PD patients display an upregulated FAS/FAS ligand system (78), potentially enhancing myeloid cell recruitment and release of cytokines (343, 344). PD patients show increased CCL2 blood levels and dysregulated $CCR2^+$ monocytes responses (78, 79). Transcriptionally, monocytes from PD patients show a specific expression profile correlating with disease severity and indicating enriched expression of genes related to migration and regulation of inflammation (345).

Blood monocytes with pro-inflammatory features are also increased in PD animal models (346) and seem to infiltrate the degenerating substantia nigra by crossing the BBB (347, 348). Accordingly, $CCR2^+$ MdMs can be found in the brain of PD mouse models early in the disease process, with astrocytes being the main producer of CCL2 (51). Of note, while blocking monocyte recruitment had no effect on dopaminergic neuron survival, overexpression of CCL2 in astrocytes did increase neuronal death and led to augmented infiltration of $CCR2^+$ monocytes, together suggesting that MdMs contribute to neurodegeneration (51). Notably, in the SNpc of a different transgenic PD model, MdMs vastly outnumbered microglia and could be engineered as a "Trojan horse" approach to locally deliver neuroprotective factors (349).

While these studies imply trafficking of monocytes across the BBB during PD, the potential contribution of meningeal and ChP gateways to cell recruitment has never been addressed in the literature. In summary, several evidences point toward an involvement of recruited monocytes to PD pathogenesis, setting the ground for future studies finally testing the importance and the therapeutical value of these cells in PD.

Alzheimer's Disease

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by brain atrophy, synaptic loss, extracellular deposition of amyloid-β (Aβ) peptides and intracellular accumulation of neurofibrillary tangles of phosphorylated Tau protein (349–351). The controversial contribution of microglia to AD pathogenesis is discussed by a growing body of literature,

with molecules such as CX3CR1, APOE and triggering receptor expressed by myeloid cells 2 (TREM2) showing a critical impact on disease evolution by regulating phagocytosis and anti-inflammatory signaling in macrophages/microglia (352–355).

In parallel, some studies have highlighted the multifactorial contribution of BAMs to AD. Aβ tends to accumulate with age in insoluble depositions limiting drainage along perivascular pathways and typically resulting into cerebral amyloid angiopathy (104, 356). PvMs importantly participate in clearing perivascular Aβ, as shown in different mouse models of AD in which ablation of pvMs resulted in augmented Aβ accumulation (88, 357). PvMs Aβ clearance depended on expression of the scavenger receptor class B type 1 (55) and of CCR2 (56). These pvMs also express CD36 (57), one of the main receptors for Aβ (36). Notably, some studies have demonstrated a CD36-dependent, *Nox2*-mediated production of ROS in response to Aβ phagomacrocytosis in pvMs, a phenomenon ultimately augmenting vascular pathology and cognitive dysfunctions (358, 359). Taken together, pvMs seem to play both beneficial and detrimental roles in AD models, however, their function in AD patients remains undefined.

The complex equilibrium between fluid drainage and Aβ deposition observed at the perivascular level in AD is also detected within the ChP. Aβ transporter proteins are expressed at both the BBB and BCSFB contributing to a coordinated clearance of Aβ into the peripheral circulation (123, 360, 361). Underscoring disease-specific defects in barrier transport mechanisms, ChP tissue from AD patients shows substantial deposition of Aβ peptide and increased oxidative stress (362). Within the ChP of AD patients, dense fibrillary phosphorylated Tau can also be shown in calcified intracellular inclusion in the proximity of $TREM2^+$ stromal ChPMs (58, 80). These events lead to significant changes in BCSFB permeability (130, 363) and to a reduced ChP expression of trafficking and inflammatory molecules including ICAM-1, VCAM-1, CXCL10, CCL2, and IFNγ, suggesting impaired monocyte migration *via* the BCSFB and a exacerbatory impact on disease evolution (364).

Concerning meningeal macrophages, one recent report has suggested a contribution of these BAMs to Aβ pathology, yet only within the dura mater and following experimental ablation of lymphatic vessels leading to local accumulation of Aβ and of $Iba1^+$ macrophages (365).

While the role of BAMs in AD remains underinvestigated, the impact of MdM on disease development has been addressed in several studies. *In vitro*, monocyte trafficking at the BBB drastically increased in the presence of Aβ and was mediated by receptor for advanced glycation end products (RAGE) and PECAM-1 expressed on endothelial cells (366). Furthermore, both mouse and human microglia stimulated with Aβ upregulate their expression of CCL2, CCL3, CCL4, and CXCL2, suggesting a substantial Aβ-dependent recruitment of immune cells to diseased brains (367–369). Accordingly, different $CD45^{high}CD11b^{high}CCR2^+$ macrophages accumulate in the brain of animal models of AD, with CCR2 deficiency leading to the detrimental accumulation of Aβ (59, 370). These earlier results indicated that MdMs might exert a beneficial effect in the disease process (370, 371). Accordingly, parenchymal $CD163^+$ microglia-like MdMs were described in the brain of AD patients in association with Aβ deposition nearby the damaged BBB (77). Unfortunately, in most of these reports, full body irradiation paradigms were utilized (372), artifactually leading to long-term changes in glia activation and increase in myeloid cell recruitment (56, 373). By shielding the mouse brain during irradiation, it was subsequently shown that $CCR2^+$ MdMs only rarely infiltrate the CNS, thus redimensioning their role in AD (56). This work also contradicted earlier results (367) by showing that microglia accumulation close to Aβ does not strictly depend on the CCL2 system (56).

Nevertheless, monocytes might contribute to Aβ removal without leaving the vascular tree. A recent study has illustrated the role of $CX3CR1^+Ly6C^{low}$ patrolling monocytes in crawling in $A\beta^+$ brain veins and engulfing intraluminal Aβ (54). However, contradictory observations have been made in AD patients, showing that circulating monocytes express reduced TLR levels (374), are defective in Aβ phagocytosis (375, 376) and are more prone to apoptosis compared to monocytes isolated from control patients (376).

In conclusion, in both mouse models and AD patients monocyte/MdM functions remain somewhat elusive. Studies on BAMs have, however, highlighted their complex role in Aβ removal and thus potentially suggested these cells as future targets of therapeutical interventions.

CONCLUSION AND OUTLOOK

Aided by stimulating debates on functional CNS anatomy (13, 97, 106, 182), by technical advancements in reporter tools and imaging (14, 44, 131) and by the recent "single-cell analysis revolution" (16, 27, 28), the study of CNS borders finally bloomed as a research field.

Long-lived dwellers of these CNS interfaces, BAMs mediate systemic communication (4), regulate vascular permeability (91, 92), waste clearance (94), fluid drainage (88), and surveil CSF composition (34). Often ignored and improperly ontogenically and anatomically defined, BAMs can now be precisely identified in contrast to blood-borne myeloid cells (14–16, 20, 22, 23), a necessary advancement allowing to address specific cell functions upon CNS damage. In this context, each CNS interface becomes a complex battleground hosting a myriad of peripherally derived trespassers. Even though the multidimensional interplay between invading monocytes, BAMs and the cellular components of each CNS gateway serves as a key checkpoint in disease evolution, a convincing picture of macrophage dynamics at CNS borders is far from existing.

What is the contribution of BAMs to pathogenesis of CNS disease?

During autoimmune neuroinflammation, BAMs increase in density, contact invading leukocytes and produce inflammatory cytokines and chemokines (42, 112, 225, 260). Surprisingly, however, these cells modify their overall transcriptional profile only mildly compared to steady state (27). Upon TBI/SCI, BAM actions seem beneficial but at the same time negligible (48, 49, 74,

294). The same applies to PD (52, 53, 76), while AD studies conversely indicate a disease-shaping role of BAMs (88, 357, 358, 365). Notably, the discussed results and recent data from brain tumor models (24) seem to indicate a relatively minor contribution of BAMs to the evolution of most CNS dysfunctions.

Conversely, during neuroinflammation, MdMs outnumber BAMs at CNS interfaces and drive disease evolution (15). Molecules such as GM-CSF (149, 229), CCL2 (240, 243–246), and different MMPs (169) primarily orchestrate the overall immigration of these cells into the CNS parenchyma, but the actual route of invasion through different CNS gateways remain remarkably speculative. Monocyte recruitment upon CNS trauma shares similar mechanisms with MS/EAE including the role of CCL2 and the need for MMP production by infiltrating cells (297, 298, 377). Lastly, monocyte trafficking to the CNS during neurodegenerative disorders remain surprisingly understudied, and its dependency on the CCL2-CCR2 axis controversial (51, 56).

To summarize, despite various efforts to understand the functional contribution of myeloid cells to CNS diseases, this review underlines how the study of BAMs and of monocytic invasion pathways is still at its infancy. Recent technical advancements should finally allow understanding whether BAMs, as long-lived dwellers of CNS interfaces, can become relevant therapeutic targets to manipulate CNS dysfunctions. Secondly, a renewed focus on CNS anatomy and barrier functions will hopefully prompt scientists to investigate and describe invading trajectories of monocytes/MdMs in the different disease models. A detailed definition of the infiltration routes and of the polarizing influence of distinct CNS gateways on trespassing monocytes will eventually allow designing targeted strategies to regulate monocyte entry, thus modulating the evolution of CNS pathologies.

AUTHOR CONTRIBUTIONS

JS designed the figures. DI prepared the tables. DI, SW, KB, and GL wrote the manuscript. All authors contributed to the article and approved the submitted version.

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The Impact of IgA and the Microbiota on CNS Disease

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Although anatomically distant from the central nervous system (CNS), gut-derived signals can dynamically regulate both peripheral immune cells and CNS-resident glial cells to modulate disease. Recent discoveries of specific microbial taxa and microbial derived metabolites that modulate neuroinflammation and neurodegeneration have provided mechanistic insight into how the gut may modulate the CNS. Furthermore, the participation of the gut in regulation of peripheral and CNS immune activity introduces a potential therapeutic target. This review addresses emerging literature on how the microbiome can affect glia and circulating lymphocytes in preclinical models of human CNS disease. Critically, this review also discusses how the host may in turn influence the microbiome, and how this may impact CNS homeostasis and disease, potentially through the production of IgA.

Keywords: IgA, gut microbiome, ageing, multiple sclerosis, neurodegeneration

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INTRODUCTION

Complex diseases of the central nervous system (CNS) are caused by a combination of genetic and environmental factors. Human studies and animal models have demonstrated that commensal microbes residing in a host can influence CNS disease (**Figure 1**), adding additional complexity to unraveling the etiology of these diseases. While cheaper and more efficient sequencing technologies has facilitated the human microbiome project (24), we are only at the beginning of identifying disease *vs.* health-promoting microbes, the environmental and genetic factors that promote such communities, and the functional output of these communities.

Two main factors may modify microbiota. First, the microbiota is highly context-dependent and modulated by geographic location and diet (25). Strong evidence suggests diet-based alterations in the microbiome come from extreme diet changes (26) or adoption of new cultural dietary habits (27, 28). There is increasing appreciation for the role of diet-microbiome interactions in CNS diseases (29). Second, internal factors such as host genetics, age, and sex are also important determinants for selecting the gut microbiota (30).

Herein we review emerging literature linking host-microbiome interactions to lymphocytes and glial cells in the context of CNS diseases. We describe two key host factors, intestinal IgA and ageing, that have a profound impact on shaping the microbiome. We also provide examples for how these factors impact lymphocytes and glial cells in the context of CNS disease. In summary, we provide a working model for how interactions between host factors (IgA and ageing) contribute towards

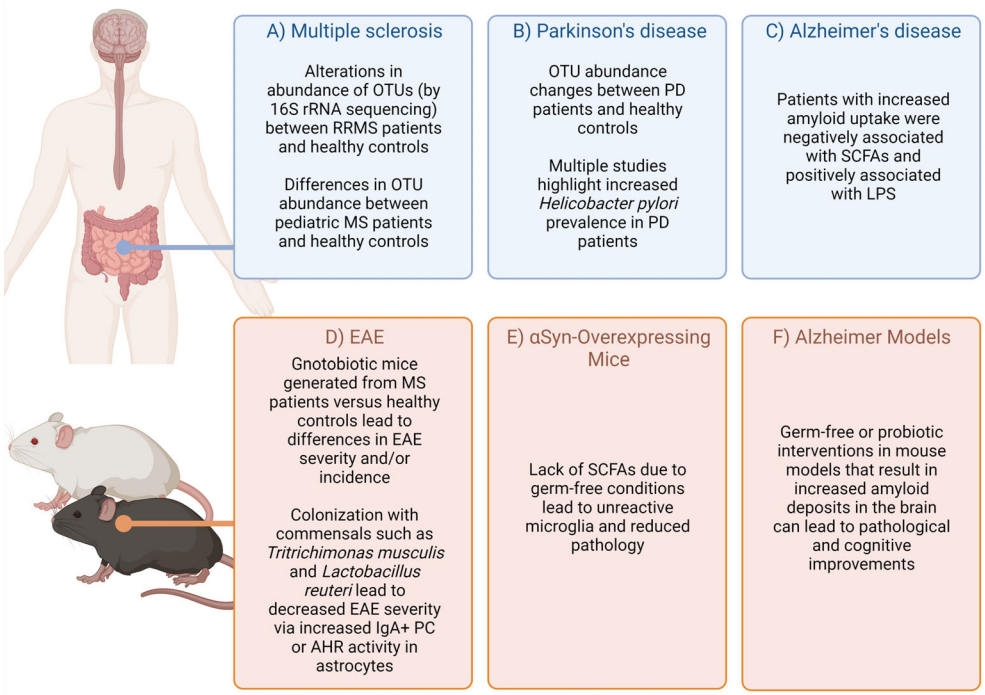

FIGURE 1 | Highlighted evidence for the relationship of microbiota affects in CNS-specific human diseases and animal models. Complex diseases of the CNS are often difficult to query in humans due to scarcity of tissue samples. However, combining evidence from patients, healthy controls **(A–C)** (1–14), as well as animal models **(D–F)** (15–23) can provide some suggestive evidence on how the microbiota may impact disease. Figure made using (BioRender.com).

shaping the microbiome which in turn can influence lymphocyte and glial cell behavior in the context of CNS disease (**Figure 2**).

INFLUENCE OF THE MICROBIOTA ON LYMPHOCYTES IN CNS DISEASE

Correlative data in MS and mouse models demonstrate a bidirectional interaction between the gut and the CNS (15, 16, 31–33); identifying specific contributions of the gut microbiome to CNS disease is imperative for understanding disease pathogenesis. While aberrantly activated lymphocytes are a hallmark of multiple sclerosis (MS), this is less studied in Alzheimer's and Parkinson's disease (AD, PD). Thus, this section will focus on the impact of microbiota on lymphocyte function in MS.

T Lymphocytes

Although alterations in the microbiome have been reported in MS case-control studies (34), testing causal associations between these alterations and disease risk requires animal models. Germ-free (GF) mice lack commensal microbiota and thus present a "blank slate" for exploring the impact of commensal microbes on disease. GF mice fail to develop experimental autoimmune encephalomyelitis (EAE) (35), but when gnotobiotically recolonized or monocolonized with segmented filamentous bacteria (SFB), EAE is rescued. SFB enhances T_H17 cell induction (36), T cells that are critical in causing pathology in EAE. Regulatory T cells (Tregs) are similarly sensitive to the gut microbiome. Their polarization from naïve T cells can be potentiated by *Bacteroides fragilis* polysaccharide in EAE (17, 35). Interestingly, following transplantation of human MS stool samples into mice (fecal microbiome transplant; FMT), several bacterial species were linked to alterations in T_H1 and Treg differentiation, and consequently EAE phenotype (37, 38).

B Lymphocytes

B cells produce antibodies, present antigen to T cells, and secrete cytokines. When antigen binds to a B cell receptor, these antigen-specific B cells are activated and undergo somatic hypermutation and affinity maturation in germinal centers (GC) (39), generating high-affinity antigen-specific receptors. GC B cells can also class-switch to generate different antibody isotypes with specialized effector functions (IgA, IgE, IgG). Mature B cells can also differentiate into memory B cells or antibody-secreting cells (ASCs) (40). ASCs comprise both proliferative plasmablasts (PBs) and terminally differentiated plasma cells (PCs) (39, 41). Alterations in microbial abundance have been correlated with changes in regulatory B cell (Breg) induction (18, 42). Antibiotic treatment enhanced frequencies of IL-10-producing Bregs at baseline and in EAE (42). In addition, a role for gut-derived commensal-reactive IgA^+ ASCs in attenuating EAE and possibly also MS has been shown (18, 19), described below.

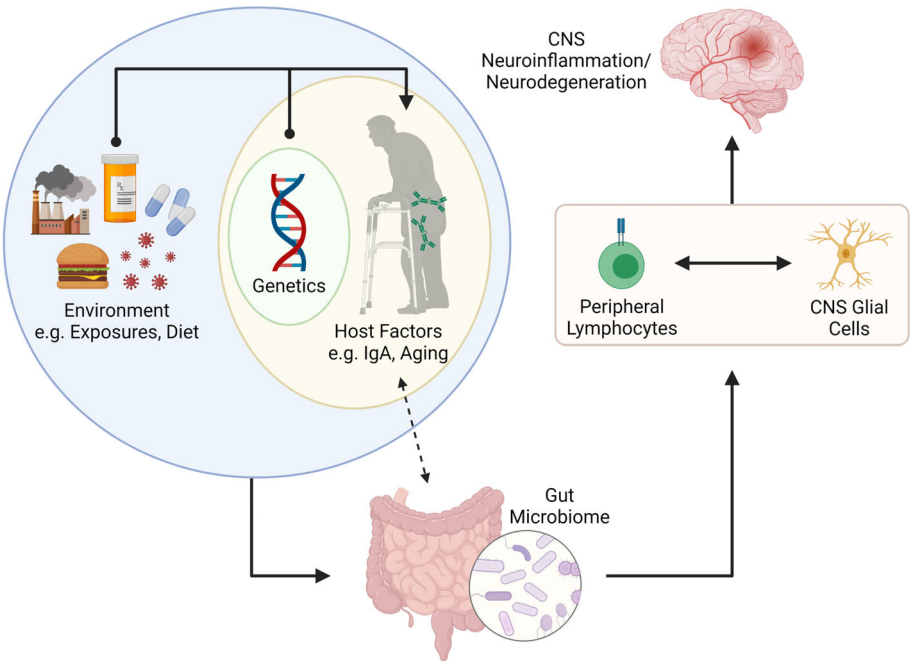

FIGURE 2 | Putative connection between the gut microbiome and CNS neuroinflammation and neurodegeneration. The gut microbiome is shaped by internal (e.g., genetics and other host factors such as mucosal IgA and age) and external factors (e.g., environmental exposures, infections, diet, etc.). Recent literature has suggested that IgA plays a key role in determining the microbes that reside in the gut, but that IgA levels can also be influenced by colonizing microbiota. The gut microbiome is important for programming of peripheral lymphocytes but can also impact the phenotype and function of CNS resident glial cells (*via* metabolites such as SCFAs. The activation (or modulation) of lymphocytes and glial cells can lead to neuroinflammation or neurodegenerative disorders in the CNS. Figure made using (BioRender.com).

INFLUENCE OF THE MICROBIOTA ON GLIAL CELLS IN CNS DISEASE

Glial cells have been intensively studied in each MS, AD, and PD, and a role for the microbiome in modulating glial cell phenotype and function in these diseases is emerging.

Microglia

Microglia are CNS-resident macrophages serving key homeostatic and immune functions in the developing and adult CNS (1). Maternal microbiota can influence microglial maturation and function during both fetal development and in adulthood, as demonstrated in GF and antibiotic-treated mice (20, 21). Given the lack of evidence for a CNS microbiome, presumably microbiota-derived metabolites, such as short-chain fatty acids (SCFAs), directly or indirectly influence microglial phenotype (43). Microglia in GF mice are not fully mature, and interestingly, colonization with altered Schaedler's flora failed to rescue microglial defects whereas SCFA supplementation reversed the abnormal phenotype (21), indicating that the presence of SCFA-producing bacteria or a diverse microbiota are necessary for maturation.

In models of MS, antibiotic treatment prior to lysolecithin (LPC)-induced demyelination decreases microglia activation, indicated by the reduction in intralesional P2ry12^{lo}Clec7a^{+} inflammatory cells as opposed to P2ry12^{+}Clec7a^{-} homeostatic microglia. Microglial activation was also attenuated in GF mice given the demyelinating toxin cuprizone (44). Surprisingly, supplementing aged mice with probiotic VSL#3 enhanced serum and fecal SCFAs, but had limited effect post-LPC administration (44). Indoxyl-3-sulfate (I3S), a product of microbial tryptophan metabolism, activates aryl hydrocarbon receptor (AHR) on microglia, augmenting TGFα production to limit astrocytic inflammation in EAE (45). Feeding whole milk promotes AHR ligand and SCFA production and ameliorates EAE in marmosets, although the effect is not attributed solely to microglia, but overall modulation of inflammation (46).

In α-synuclein-overexpressing (ASO) mice that model PD, the presence of a gut microbiota promotes the aggregation of α-synuclein in the caudoputamen and substantia nigra, resulting in microglial activation and motor dysfunction (47). GF ASO mice show significantly decreased levels of aggregated α-synuclein and are protected from development of motor deficits. Re-colonization of GF mice with SPF microbiota reversed this rescue effect. Surprisingly, despite SCFAs being generally thought to be anti-inflammatory, this study inculpates SCFAs in promoting aggregated α-synuclein. SCFA supplementation to GF or antibiotic-treated ASO mice increased microglial activation and is sufficient to promote motor impairment. In addition, abundance of several SCFA-producing enzymes is increased in humanized mice that have received an FMT from Parkinson's disease donors.

Like other neurodegenerative diseases, an unhealthy microbiome is increasingly appreciated as a risk factor for AD.

Similar to PD, circulating levels of the SCFAs acetate and valerate were positively correlated with Aβ load in the brains of AD patients (48). Mice treated with probiotic bacteria exhibited ameliorated AD-like cognitive decline and decreased Aβ aggregation (49). GF 5x Familial Alzheimer's disease (5xFAD) mice show a decreased Aβ load in the hippocampus compared to conventional 5xFAD mice, attributed to the uptake of Aβ debris by microglia. A greater number of $Iba1^+$ cells were found in the hippocampus closely associated with Aβ plaques in GF 5xFAD mice, and a higher percentage of these plaque-associated $Iba1^+$ cells were positive for methoxy-X-O4, an indicator of Aβ uptake (50).

Astrocytes

Astrocytes play diverse roles in the homeostatic brain that include providing trophic support to other CNS cells, regulation of synaptic activity, and controlling the blood-brain barrier (51–54). Astrogliosis is also a feature of MS. Unsurprisingly, astrocytes play important roles in modulating neuroinflammation, as they can produce inflammatory cytokines and a host of chemokines that promote chemotaxis of other immune cells. In EAE, astrocytic inflammation is shown to be directly attenuated by I3S activation of AHR (45). Gut microbiota depletion by antibiotic treatment decreases levels of I3S and worsens EAE disease (55). AHR-deficient astrocytes increase expression of several pro-inflammatory chemokines and cytokines. Importantly, IFNβ, a therapeutic used in some MS patients, works to limit CNS inflammation through this mechanism, as the anti-inflammatory effect of IFNβ is lost in AHR-deficient astrocytes (55).

Oligodendrocytes

Oligodendrocytes were previously thought to be quiescent, myelin-producing cells. However, increasing evidence shows that oligodendrocytes actively communicate with and provide metabolic support to neurons (56). Mature oligodendrocytes may also participate in remyelination and are active players during neurodegeneration (57). However, little is known about interactions between oligodendrocyte lineage cells and the gut microbiome. While treatment of mice with the probiotic VSL#3 enhanced SCFA concentrations in feces and serum, there was no effect on remyelination *in vivo* following LPC-induced demyelination (44). Conversely, a separate study found that in *ex vivo* organotypic cerebellar slice cultures demyelinated by lysolecithin, the addition of the SCFA member butyrate enhanced both OPC numbers and mature oligodendrocyte numbers, indicating a positive effect on remyelination (58). In summary, the gut microbiota exerts effects not only on peripheral immune compartments, but also act on glial cells, with potential impacts on CNS disease processes.

HOST FACTORS THAT INFLUENCE THE INTESTINAL MICROBIOME – A FOCUS ON IGA AND AGEING

Many external factors influence the gut microbiome such as diet and pathogen exposure. In this section, we review host factors that shape the microbiome, focusing on IgA and ageing.

Host Control of the Microbiota Through IgA

Mature B cells primed in Peyer's patches can differentiate into IgA-producing PCs and home to the intestinal lamina propria (41, 59, 60). The IgA produced by GALT PCs is typically dimeric, joined through the J-chain (41, 61). Secretory IgA is generated when dimeric IgA binds the polymeric-Ig-receptor (pIgR) on the basolateral surface of the intestinal epithelium, translocates through the epithelial cells, and is released into the lumen with the secretory component of the pIgR.

In mice, IgA both contributes to host control of microbiota and is responsive to gut microbiota changes. Mice monocolonized with *Bacteroides thetaiotamicron* harbor a reduced IgA repertoire restricted to a single clone against the capsular polysaccharide of the bacterium (62). Oral administration of *Lactobacillus casei* to mice resulted in an increase in IgA^+ cells in the small intestinal lamina propria (SILP) (63). Exposure of conventional mice to commensal Proteobacteria also resulted in increased serum IgA levels and induction of IgA^+ PC in the bone marrow (64). In contrast, some microbial communities can diminish IgA levels in the lumen due to their ability to degrade the secretory component (65). Even strain level differences in the microbiome can dictate IgA levels in the host (66). Conversely, the host IgA response can dictate the composition of the microbiome. Activation-induced cytidine deaminase-deficient mice (which fail to produce competent IgA), exhibit an expansion of SFB in the small intestine which leads to isolated lymphoid follicle (ILF) hyperplasia and GC enlargement in secondary lymphoid tissues (67, 68). Restoration of IgA by heterogenetic parabiosis with wild-type mice reduced SFB populations, ILF protrusion, and spleen and lymph node size.

In humans, modest alterations in fecal microbiota composition are observed in subjects with selective IgA-deficiency (SIgAd) (69, 70). Compensatory sIgM in SIgAd subjects has a distinct bacterial binding pattern: an unclassified *Enterobacteriaceae* taxon heavily coated by IgA in healthy controls and by IgM in selective IgA-deficient subjects, was significantly more abundant in SIgAd subjects, demonstrating that IgA coating specifically restricts expansion of this taxon, and the same effect is not achievable by IgM.

Overall, these data indicate that in both mice and humans, a bi-directional relationship exists between host IgA and gut microbiota.

Impact of Ageing on the Microbiome

Growing evidence suggests that the gut microbiota has a critical impact on the ageing process and is a possible determinant of healthy ageing (71–73). Cross-sectional studies have examined alterations in the microbiota composition across the human lifespan (74, 75), demonstrating that taxonomical composition of gut microbiota appears to follow stepwise progression through life. Taxonomic shifts in the microbiota and decrease in microbial richness and diversity are observed in frail older individuals and associated with worse health outcomes compared to younger individuals (76–80). Relative abundance of Ruminococcaceae, Lachnospiraceae, and Bacteroidaceae families decrease with age, whereas an enrichment and/or higher prevalence of health-associated genera such as

Akkermansia, *Bifidobacterium*, and Christensenellaceae, are maintained in longevity and extreme longevity (81). Indeed, centenarians tend to exhibit indicators of good health, and greater gut microbiota complexity (74). The relative abundance of pathobionts decreases and beneficial commensals, such as *Akkermansia*, are retained (82). Studies that stratify between elderly and centenarian status identify changes in taxa associated with extreme longevity including *Odoribacter*, *Butyricimonas*, *Desulfovibrio*, *Bilophila*, *Oscillospira*, and *Akkermansia* genera, and the Christensenellaceae and Barnesiellaceae families (75, 81).

Similar taxonomic and functional patterns that correlate with age and frailty in the mouse microbiome have been identified (83). In aged mice, the ratio of Firmicutes to Bacteroidetes increased ∼9-fold compared to young mice, indicating dysbiosis, although this work was performed in commercially purchased mice rather than mice derived from the same dam (84). Introducing aged microbiota to young mice increased mortality following ischemic stroke, decreased behavioral outcomes, and increased cytokine levels. Conversely, altering the microbiota in aged mice to resemble that of young mice increased stroke survival and improved recovery (84). Changes in the gut microbiota in aged mice were also associated with increased gut permeability and elevations of peripheral inflammation (85, 86). Taken together, except for healthy centenarians who resist frailty, ageing is associated with an unhealthy microbiome.

INFLUENCE OF AGEING AND IGA ON CNS DISEASE *VIA* THE MICROBIOME

Multiple internal host factors impact the microbiome, including IgA and ageing. Here we speculate on how these two host factors may impact brain health and the trajectory of brain disease *via* the microbiome.

IgA, the Microbiome, and CNS Disease

Although IgA^+ ASCs can home to the dura mater during homeostasis (87), clonally expanded IgA are absent in steady state CNS and only appear during inflammation (88–91). During EAE, a significant reduction in IgA^+ ASCs is apparent in the SILP. Additionally, adoptively transferred gut-derived IgA^+ ASCs were found in the CNS were reactive to mouse-derived gut bacteria and were shown to alleviate neuroinflammation by producing IL-10 at chronic stages of EAE. Over-abundance of IgA^+ ASCs was able to reduce GM-CSF production by T cells, an important cytokine that promotes neuroinflammation (18). *Tritrichomonas musculis* (*T.mu*) is a rodent commensal that promotes IgA production (92). EAE incidence and severity, as well as spinal cord inflammation and demyelination, are reduced in $T.mu^+$ mice (18). $T.mu^+$ mice also exhibited elevated serum and fecal IgA levels and increased frequencies of IgA^+ ASCs in the gut, bone marrow, and brain.

While the above highlights key findings from animal models, there is also early evidence suggesting the importance of the microbiota-driven IgA response in human disease. Bacteria identified by IgA-seq were differentially expressed in MS patients *versus* healthy controls (19). Stratified by disease activity, MS patients in relapse exhibited decreased percentages of IgA-bound gut bacteria in fecal samples compared to remitting patients, with corresponding elevation in CSF IgA. CNS-infiltrating IgA^+ B cells show specificity for gut microbial antigens, indicating the migration of IgA-producing cells from the gut during relapse. IgA is also elevated in cerebrospinal fluid of MS patients. Importantly, commensal-specific IgA^+ ASCs have been observed in inflammatory lesions of MS patients (19). This phenomenon may not be IgA-exclusive, however, as IgG in MS patient CSF has been found to be reactive against MS-associated gut bacterial lysate (93). The implications of these bacteria-reactive IgG in disease have yet to be fully elucidated.

Lastly, while IgA^+ ASC have been now described in the inflamed EAE and MS CNS, it is now appreciated that these cells play an important role in homeostasis. Specifically, intestinal commensal specific IgA^+ ASC have been detected in the leptomeninges of healthy mice and humans but are absent in GF mice (87). These cells likely maintain barrier integrity near the dural sinuses; however, it is possible they may also contribute to quiescence within the CNS.

In summary, in addition to its well appreciated role in shaping the microbiome, IgA-producing ASC likewise play important roles in the healthy and MS/EAE CNS. The role for these cells in PD and AD is not yet understood.

Ageing, the Microbiome, and CNS Disease

Ageing is the predominant risk factor for neurodegenerative diseases (94), yet in spite of the known role ageing has on the microbiome, the connection between ageing, the microbiome and CNS disease has barely been explored.

It is well established that microglia are affected during ageing. Ageing results in decreased number, uneven distribution, lower motility, and fewer ramifications, as well as impairment in phagocytosis and injury responses (2, 95–98). Senescent microglia increase pro-inflammatory cytokine production (3). An altered microglia morphology and reduced arborization have been observed in the human brain during ageing and age-related diseases such as AD (95). This dystrophic morphology is associated with impaired spatial learning (3).

Age-related changes in the gut microbiome may have a direct impact on microglial function within the CNS. In fact, reduced complexity of microbiota, a feature of ageing, leads to defects in microglia maturation and function (21). Recent work demonstrated that FMT from aged donor mice into young recipients impairs spatial learning and memory in young recipients (4). Conversely, FMT from young donor mice into aged recipients can rejuvenate age-associated CNS metabolic, transcriptomic, and behavioral changes (5). Aged into young FMT induced an altered expression of proteins involved in synaptic plasticity and neurotransmission in the hippocampus, an area of the CNS known to be affected by the ageing process. A strong reduction of bacteria associated with SCFA production (Lachnospiraceae, *Faecalibaculum*, and Ruminococcaceae) and

disorders of the CNS (Prevotellaceae and Ruminococcaceae) was also reported (4). Interestingly, microglia of the hippocampus acquired an ageing-like phenotype following FMT. Of therapeutic relevance, this age-associated phenotype can be reversed by re-introducing live and complex microbiota or microbial metabolites, such as SCFAs (6).

The gut microbiota similarly affects astrocytes in both ageing and age-associated neurodegenerative diseases (7). Ageing can alter the normal function of astrocytes which reduces their ability to properly maintain a healthy CNS environment (8). Astrocyte transcriptomes from multiple mouse brain regions have revealed that ageing upregulates genes that eliminate synapses and induces a reactive astrocyte gene signature (9). Therefore, aged astrocytes may promote synapse elimination and neuronal damage, contributing to ageing-associated cognitive decline. Morphological changes in astrocytes have also been documented in the aged CNS (10–12). Aged astrocytes increase cytokine production, notably CXCL10 (13) that attracts peripheral immune cells and promotes T cell adhesion to endothelial cells (14). CXCR3, which is the CXCL10 receptor, is expressed in microglia, suggesting that astrocytes and microglia communicate during ageing (22, 45).

CONCLUSIONS

Chronic, complex diseases of the CNS develop over years. Animal studies conducted under controlled conditions in short periods miss two large confounders in these diseases – time (ageing) and the microbiota-IgA axis, with age-associated microbiota alterations further complicating this relationship. These are important considerations for animal modelling, given the considerable variability in microbiota composition and gut luminal IgA levels between vivaria (65). In summary, we propose that host factors such as age and intestinal IgA are key determinants in how the microbiome impacts lymphocyte and glial cell phenotype/function in the context of MS, AD and PD (**Figure 2**).

AUTHOR CONTRIBUTIONS

AP, DL, BI, and IN all contributed to the writing of this manuscript. AP, DL, and JG contributed to the editing of the text and generation of all figures. All authors contributed to the article and approved the submitted version.

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