Investigating the role of microglia in myelin development

By

Liang Li
B.Eng. Chemical and Biomolecular Engineering
National University of Singapore, 2014

Submitted to the Institute for Data, Systems, and Society
in partial fulfillment of the requirements for the degree of
Master of Science in Technology and Policy
and
Master of Science in Electrical Engineering and Computer Science
at the

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Abstract

We investigate the role of microglia in myelin development. Myelination is an essential process in early development that consists of the ensheathment of axons by myelin, which ensures rapid conduction of action potentials. Although myelination is predominantly driven by oligodendrocytes, the other glial cells, including microglia also contribute to this process. Microglia are resident immune cells in the central nervous system (CNS) and carry out important functions not only in injury and disease but also in homeostatic conditions. While the role of microglia in myelination has been explored by previous studies, little is known about the precise mechanism. Recently, a distinct microglia subset characterized by high expression of *Spp1, Gpnmb*, and *Igf1* was found in white-matter regions in the early post natal brain but not at other time points. First, we developed a novel constitutive Cre mouse line, Fcrls-Cre, using the CRISPR-Cas9 system to target all subsets of microglia, including the white matter-associated microglia. Second, we focused on a signaling pathway triggered by ligand IgG and the gamma chain of its receptor, FcRγ, and investigated their roles in the development of myelin. Our study hopes to provide a valuable tool to study microglia *in vivo* and to increase the understanding of how microglia contributes to myelin.

In the Appendix, I present a brief review of the promises and challenges of CRISPR in gene therapy in hopes to inform the discussions on the economic, ethical, and regulatory implications of gene editing.

Thesis Supervisor: Guoping Feng
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Acknowledgements

When I first entered MIT, I would have never thought that I would work on neuroscience research that this thesis is based on. Two years into the program, I decided to change labs to explore and develop my interests in neuroscience. With an email exchange and a brief meeting, Professor Guoping Feng took me in as a summer student in his lab. It was the summer of 2019 when I was uncertain about my future career direction. I will always be grateful that Guoping put faith in me and supported me. He continuously inspires me with his vision, kindness, and lifelong dedication to research and to supporting his students.

After entering the lab, I worked closely with Tobias Kaiser, who is passionate about microglia, teaching, and kite surfing. Under his guidance, I discovered neuroscience research and experienced how fun and rewarding research can be for the first time despite the difficulties and frustrations that come with it. Tobias' mentorship is exemplary in the combination of concrete feedback, attentiveness, and patience. I remember one time when I was learning an experiment and after three repeated trials, I still couldn't get the desired result. Patiently, Tobias chose to repeat the experiment with me, side by side, checking and correcting if I made any mistakes. It meant a lot to me. Tobias taught me how to conduct research meticulously, think critically, and push scientific boundaries with perseverance. This thesis wouldn't have been possible without his insights, advice, and edits.

My sincere gratitude also goes to Professor Thomas Heldt, who is a great teacher. I thank him, for his time and help, and for being willing to read my thesis. The course he taught, "Quantitative

Physiology" is one of my favorite at MIT and inspired me to take his second course "Cellular Neurophysiology", which helped me understand the fundamentals of neurobiology.

None of this would have been possible without the support of TPP, an interdisciplinary program that is a hidden gem at MIT. I thank Frank Field and Barb DeLaBarre for supporting me throughout my time here, for their kindness, and for approving all the courses and writing all the letters I requested, which allowed me to explore my interests at MIT and beyond. My gratitude also goes to Ed Ballo, who organized unforgettable Boston tours for us, and Noelle Selin for her excellent leadership at TPP.

I would like to thank Grégoire Jacquot, for broadening my world and for inspiring me to do what I like, which is not always obvious. Without his support, I would not have pursued the research I do now. I want to thank all the friends I made at TPP who enriched my life in ways that I would never have imagined. The moments of kindness, excitement, and laughter that the TPP community provided will always stay with me.

I also would like to thank all the members of the Feng lab. The diversity of people and their projects still amaze me. I appreciate all the help and the casual talks I had with them, which made the lab experience much more enjoyable. A thank you also goes to the entire MIT community. I had the privilege to study and work with some of the most brilliant people in the world. A humbling and empowering experience.

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Investigating the role of microglia in myelin development

1. Background

1.1 Microglia

Microglia are the resident immune cells of the brain that comprise approximately 10% of the brain cells. Microglia are activated and respond rapidly and robustly to injury and disease in the brain. The idea that microglia may play important roles in development and homeostasis is relatively new and only in recent years have we started studying these cells in the context of normal health and normal development. This increase in interest to study microglia in the healthy CNS is to some extent excited by fate-mapping studies showing the origin of microglia (Schafer & Stevens, 2015). It was initially thought that blood monocytes cross the blood-brain barrier (BBB) and give rise to microglia. However, fate-mapping studies demonstrate that microglia are not bone marrow-derived, but instead originate from myeloid progenitors in the yolk sac and arrive in the brain during early embryonic development (Ginhoux et al., 2010). Remarkably, microglia are long-lived and not replenished by peripheral cells from the circulation, at least under homeostatic conditions (Florent Ginhoux & Prinz, 2015). Given the early colonization and long life in the brain, microglia are poised to play important roles in the development of CNS and normal nervous system function.

1.2 Microglia play diverse roles in development and disease

Indeed, numerous studies have shown that microglia support neuronal development, facilitate programmed cell death, prune synapses, and regulate myelin formation (Schafer & Stevens, 2015) ***(Figure 1).*** In CNS injury and disease, microglia are the resident macrophages that clear dead or dying cells and cellular debris through phagocytosis (Sierra et al., 2013). Microglia may play an

even bigger role, initiating cell death before phagocytosis or driving apoptosis in cells that are already rendered vulnerable (Marín-Teva et al., 2004; Wakselman et al., 2008). This raises the possibility that microglia regulate neuronal cell numbers during brain development. Indeed, studies showed that microglia engulf neural precursor cells to regulate the developing brain (Cunningham et al., 2013).

Microglia also participate in synaptic pruning by engulfing and eliminating unwanted synapses in development. Schafer et al. showed that complement proteins C1q and C3 send microglia "eat me" signals and that microglia prune and eat away developing synapses tagged by C1q and C3 (Schafer et al., 2012).

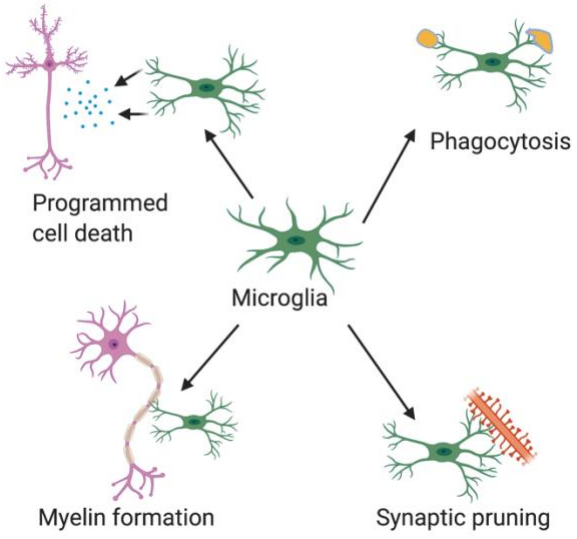


Figure 1 Microglia play diverse roles in development and disease. Created with BioRender.com

1.3 Heterogeneity of microglia

The distinct functions of microglia in development, health, and disease are also accompanied by differences in their morphology and gene expression. Earlier analyses, such as flow cytometry, in situ hybridization, or immunohistochemistry are limited to probing only a few selected RNAs or proteins at a time, which inhibits the ability to study microglial heterogeneity on a larger scale.

With recent rapid advances in single-cell RNA sequencing (scRNA-seq), a more comprehensive view of microglia heterogeneity on a single cell level has emerged, allowing us to further understand microglia diversity during development, homeostasis, and perturbation. Of note, several novel subclasses of microglia were identified at embryonic and early postnatal time points in mice (Hammond et al., 2019; Q. Li et al., 2019). A distinct microglia subset characterized by very high transcript levels of *Spp1* (encoding secreted phosphoprotein 1, also known as osteopontin), *Igf1* (encoding insulin-like growth factor 1), and *Gpnmb* (encoding transmembrane glycoprotein NMB) was frequently found in white-matter regions of the early postnatal brain (P4/P5), such as the corpus callosum and cerebellum, but was almost absent at any other time point (***Figure 2***).

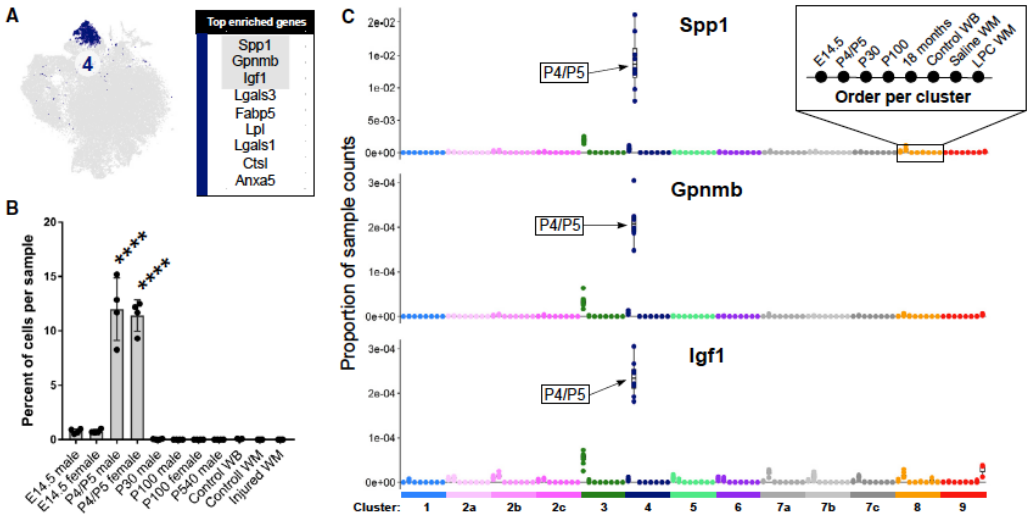


Figure 2 Distinct microglia subcluster associated with axon tracts in the early postnatal brain expresses upregulated *Spp1, Gpnmb & Igf1* (Adapted from Hammond et al., 2019) *(A) tSNE plot of cluster 4 microglia and a table of the top nine upregulated genes in that cluster. Gray outlined genes are plotted in (C). (B) Plot of the percent of cells per sample that were assigned to cluster 4. (C) Plot of the proportion of normalized UMI counts per sample for cells assigned to each cluster for the top genes in cluster 4.*

1.4 Myelination

In the CNS, glial cells called oligodendrocytes extend many processes and wrap axons to form myelin sheaths. Myelin sheaths make possible the rapid saltatory conduction of action potentials by acting as electrical insulators. Fully myelinated axons conduct impulses much faster than unmyelinated axons of the same cross-sectional size (Waxman, 1980).

Multiple sclerosis (MS) is the most common demyelinating disease of the CNS and yet the etiology is not fully understood. In addition, myelin abnormalities are also pathological features of many neurodevelopmental and neurodegenerative diseases, including autism spectrum disorder, schizophrenia, and Alzheimer's disease (Graciarena et al., 2019; Nasrabady et al., 2018; Uranova

et al., 2011). Understanding the mechanism of myelination and its regulation is critical for informing therapeutic strategies for such diseases.

Oligodendrocytes are derived from morphologically complex precursor cells (oligodendrocyte precursor cells or NG2 cells). OPCs undergo a series of steps to differentiate into premyelinating oligodendrocytes, which extend processes and finally become mature oligodendrocytes (OLs) that myelinate axons (***Figure 3***). Oligodendrocyte maturation is tightly regulated by a number of extrinsic and intrinsic factors. For instance, platelet-derived growth factor A (PDGF-A) is required for oligodendrocyte maturation in the mouse brain (Calver et al., 1998).

A major challenge in demyelinating diseases like multiple sclerosis is that populations of OPCs that preserved in demyelinated MS lesions fail to differentiate into OLs and remyelinate. Therefore, understanding the signaling pathways that lead to the differentiation of OPCs into OLs could provide insights for therapeutic approaches to regenerate myelin or prevent myelin loss in the first place.

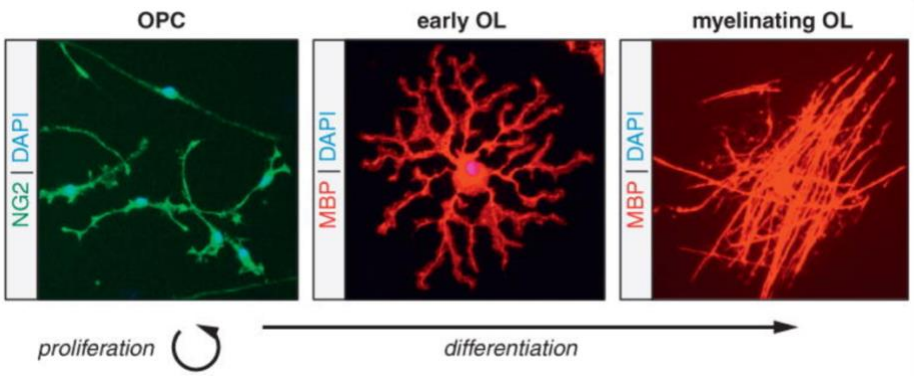


Figure 3 Fluorescence micrographs of primary cultured oligodendrocyte precursor cells (OPCs), pre-myelinating oligodendrocytes (early OLs), and oligodendrocytes (OLs) myelinating unlabeled CNS axons (Adapted from Zuchero & Barres, 2013). *NG2 (green) labels OPCs, whereas myelin basic protein (MBP, red) labels differentiated OLs and is found in compact myelin.*

1.5 Interactions between microglia and myelin - helping or engulfing?

Although myelination is predominantly driven by oligodendrocytes, the other glial cells, including astrocytes and microglia, also contribute to this process. How microglia exactly influence myelination during development remains unclear. In efforts to explain the mechanism, recent studies present two theories: one theory suggests microglia phagocytose myelin while the other supports the idea that microglia help to promote the growth of myelin (Hagemeyer et al., 2017; Hughes & Appel, 2020; Q. Li et al., 2019; Nemes-Baran et al., 2020; Wlodarczyk et al., 2017).

The theory that emphasizes the phagocytic function of microglia states that microglia engulf cells of the oligodendrocyte lineage or myelin sheaths directly to mediate myelin development (Hughes & Appel, 2020; Q. Li et al., 2019; Nemes-Baran et al., 2020). The generation of oligodendrocytes occurs during early postnatal development, coincident with neurogenesis and synaptogenesis (Kessaris et al., 2006). It could be possible that microglia-dependent homeostatic mechanisms that regulate neurons and synapses may also play a role in myelin formation. To explore this possibility, Hughes and Appel used zebrafish to visualize and manipulate interactions between microglia, oligodendrocytes, and neurons (Hughes & Appel, 2020). Because zebrafish larvae are transparent, cells of the nervous system can be followed during the course of development. They observed that microglia survey and examine myelinated axon tracts and remove excess myelin sheaths by phagocytosis. Furthermore, the amount of myelin that microglia phagocytose depended on neuronal activity, suggesting that neurons instruct microglia to remove myelin. Another study by Li et al. suggests that the group of early postnatal microglia in the developing white matter phagocytose newly formed oligodendrocytes to create a niche for more definite myelination (Q. Li et al., 2019).

The second theory posits that microglia help to promote the generation of CNS myelin via secretion of signaling molecules to support OPC survival and differentiation (Hagemeyer et al., 2017; Wlodarczyk et al., 2017). Hagemeyer and colleagues identified a subset of microglia that displayed the amoeboid morphology specifically located in myelinating regions with the expression of the activation marker Mac3 high from P1 to P8 but dramatically collapsing at P9 (Hagemeyer et al., 2017). They found that oligodendrocyte progenitor cells were strongly reduced in neonatal mice after depletion of microglia using CSF1R inhibitor BLZ 945. The effect of the early postnatal microglia on OPC numbers during development suggests that this population of microglia may mediate the survival and maturation of OPC into oligodendrocytes.

Consistent with this hypothesis, the study by Wlodarczyk suggested that insulin-like growth factor-1 (IGF-1) could be one of the molecules that induce survival of OPCs and their differentiation into oligodendrocytes (Wlodarczyk et al., 2017). Wlodarczyk and their colleagues first identified a subset of neonatal microglia that highly express CD11c, a dendritic cell surface marker encoded by the gene *Itgax*. These microglia are located predominantly in the developing corpus callosum and cerebellar white matter and this subset of microglia express significantly higher levels of *Igf1* than their $CD11c^-$ counterparts. They then conditionally deleted *Igf1* from $CD11c^+$ microglial cells and found a reduction in brain weight and significantly decreased myelination in the corpus callosum, as measured by myelin protein level (Mbp, Plp, Mag, and Mog) and myelin G-ratios in electron micrographs. Interestingly, transcriptional analysis of $CD11c^+$ microglia reveals enrichment of other notable genes such as *Spp1* and *Gpnmb*, in agreement with Hammond's and Li's single-cell analyses (Hammond et al., 2019; Q. Li et al., 2019), suggesting that this specialized

white-matter microglia type plays important roles for the proper maintenance and maturation of oligodendrocyte progenitors.

However, it is worth noting that a recent paper challenged the findings of Hagemeyer that microglial depletion through CSF1R inhibitor BLZ945 results in reduced OPC numbers (Y. Liu et al., 2019). Liu et al. argue that CSF1R inhibitor BLZ945 may directly impact OPC viability through off-target binding to PDGFRα, which is highly expressed by OPCs and is essential for their survival. Together with their own evidence, they claim that microglia are not essential for OPC viability. This prompted us to devise our own microglia depletion experiments, which are still ongoing, to decide whether microglia are essential for OPC survival, maturation, and myelination.

2. Generation of novel transgenic mouse lines for targeting white matter-associated microglia

2.1 Introduction

One of the limitations to studying microglia-specific contributions to myelination is the lack of tools to specifically delete genes only in microglia at early developmental time points. Beyond microglia, there are other macrophages in the brain. In the context of CNS injury and inflammation, circulatory monocytes derived from bone marrow invade the CNS and differentiate into macrophages (Shechter et al., 2009). In addition, there is another group of macrophages, known as CNS-associated macrophages (CAMs, also known as border associated macrophages [BAMs]) that reside in the CNS. CAMs are located at CNS-periphery interfaces such as the perivascular space, the meninges, and the choroid plexus. Recent studies with scRNA-seq analysis and mass cytometry studies described distinct signature for both microglia and CAMs including *Tmem119, P2ry12, Fcrls, SalI1, Hexb,* and others for microglia, whereas CAMs were characterized by high expression of genes including *Ms4a7, Mrc1, Pf4,* and others (Goldmann et al., 2016; Jordão et al., 2019; Mrdjen et al., 2018).

Among the identified canonical microglial genes (*Fcrls, Tmem119, P2ry12, and Cx3cr1*), *Fcrls* seems to be more uniformly expressed (***Figure 4***) and expressed at much higher levels in microglia from the developing brain compared to *Cx3cr1* and *Tmem119,* based on transcriptomic analyses (***Figure 5***). Therefore, *Fcrls* could be a potential genetic marker for microglia, especially in the early postnatal brain.

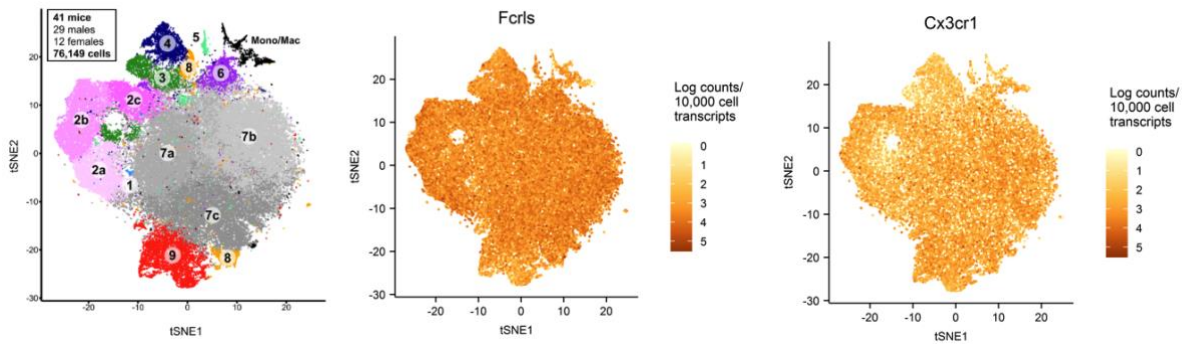


Figure 4 Fcrls is expressed homogeneously across all subsets, including the white-matter associated microglia in cluster 4. *tSNE plot of all clusters and tSNE plot of expression for Fcrls and Cx3cr1 respectively.*

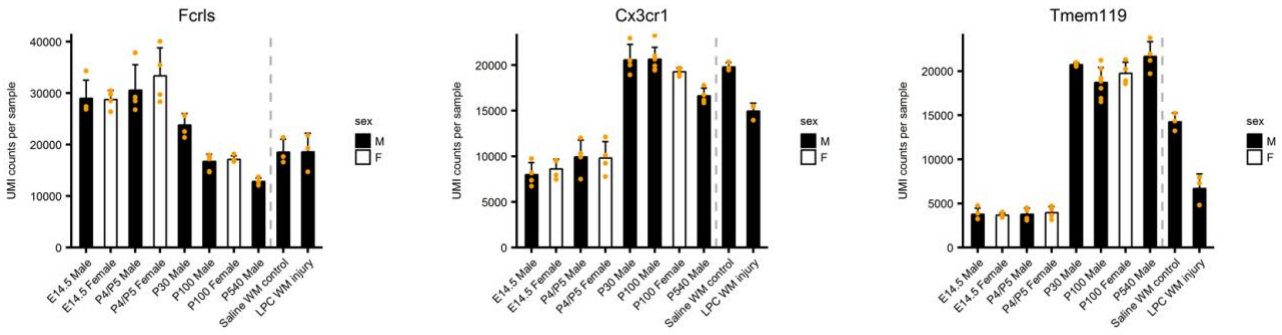


Figure 5 *Fcrls* is expressed at higher levels in microglia in the developmental stage compared to *Cx3cr1*, and *Tmem119* (Adapted from Hammond et al., 2019). *UMI counts per sample showing expression levels of Fcrls, Cx3cr1, and Tmem119 at different time points and states.*

2.2 Results

To specifically target white matter-associated microglia at the early developmental stage, we focused on the *Fcrls* locus and generated Fcrls-Cre and Fcrls-CreERT2 knock-in mouse lines using CRISPR-facilitated homologous recombination (T.Kaiser, G.Feng, unpublished). In these constructs, *Fcrls* expression was preserved by using DNA sequences encoding a ribosome

skipping 2A peptide replacing the Fcrls stop codon. 2A sequence was then followed by the coding sequences for Cre and CreERT2 (***Figure 6***).

To visualize the efficiency and specificity of Fcrls-Cre recombination, we crossed Fcrls-Cre and Fcrls-CreERT2 with Ai14 reporter mice, which express TdTomato (tdT) upon Cre-mediated recombination (Madisen et al., 2010).

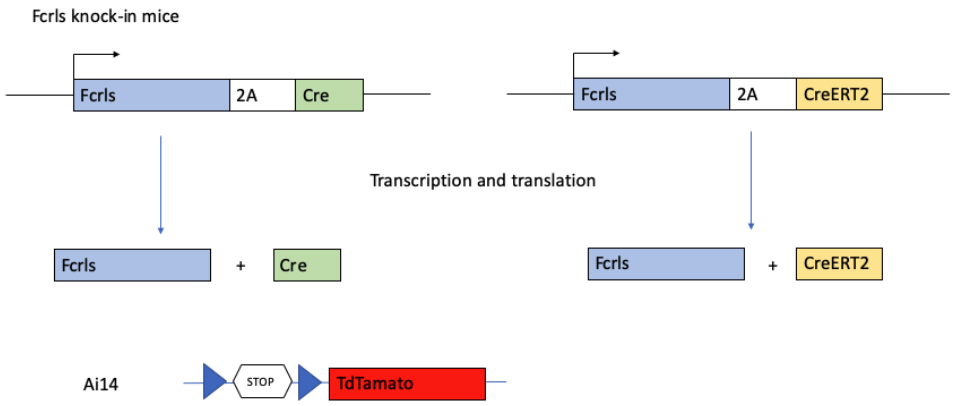


Figure 6 Schematic of Fcrls-Cre and Fcrls-CreERT2 knock-in constructs and Ai14 reporter mouse line.

To determine the efficiency and specificity of Fcrls-Cre mice, we performed immunostaining and examined microglia in several regions of the CNS using confocal microscopy on brain slices prepared from postnatal day P7 mice. We found high levels of recombination in CNS IBA1$^+$ microglia compared to control (***Figure 7***). Completion and fidelity of recombination were assessed quantitatively in the corpus callosum, cortex, and cerebellum regions of the CNS. We evaluated the completeness of labeling as the ratio of tdT$^+$IBA1$^+$ double-positive cells to all parenchymal microglia (IBA1$^+$) and fidelity of labeling as the ratio of tdT$^+$IBA1$^+$ double-positive cells to all tdT-labeled cells (tdT$^+$). Quantification showed that all IBA1$^+$ microglia across different regions coexpress tdTomato (***Figure 8*** J). We found close to 100 percent specificity in the corpus callosum and cerebellum and good specificity in the cortex (***Figure 8*** K).

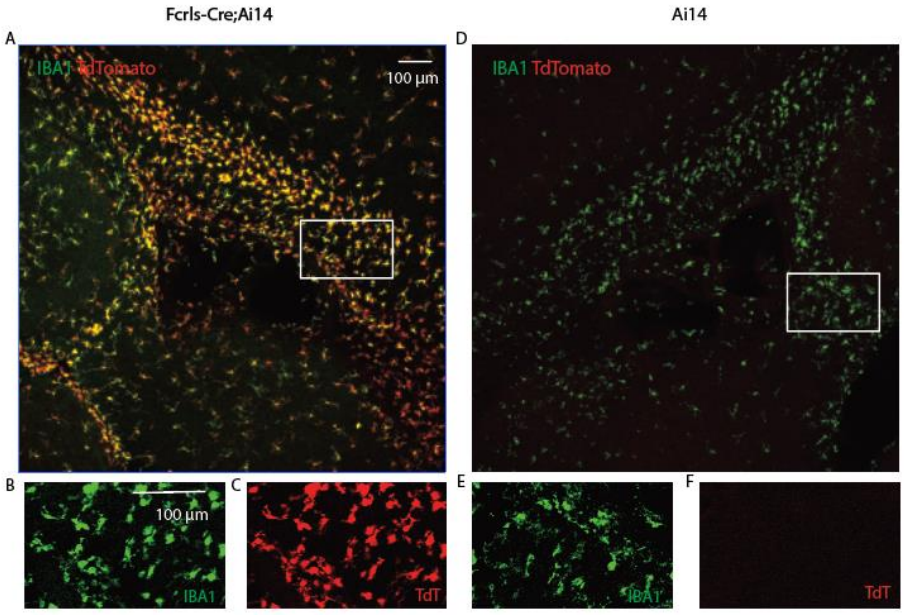


Figure 7 Successful Fcrls-Cre recombination in IBA1$^+$ microglia (T. Kaiser, L. Li, G. Feng). ***(A-C)*** *Representative confocal images showing tdTomato expression in IBA1 positive microglia in an Fcrls-Cre;Ai14 mouse* ***(D-F)*** *No TdTomato recombination in a control mouse*

As discussed, CAMs in the perivascular space, the meninges, and the choroid plexus also express marker IBA1. To determine recombination in CAMs, we examined tdT expression in the meninges and choroid plexus, which can be analyzed by their morphology, anatomical locations, and IBA1 immunoreactivity. Confocal images show that most IBA1 positive leptomeningeal (arachnoid and pia), as well as choroid plexus macrophages also express tdT, which suggests that Fcrls-Cre also mediates recombination in CAMs (***Figure 8*** A-F).

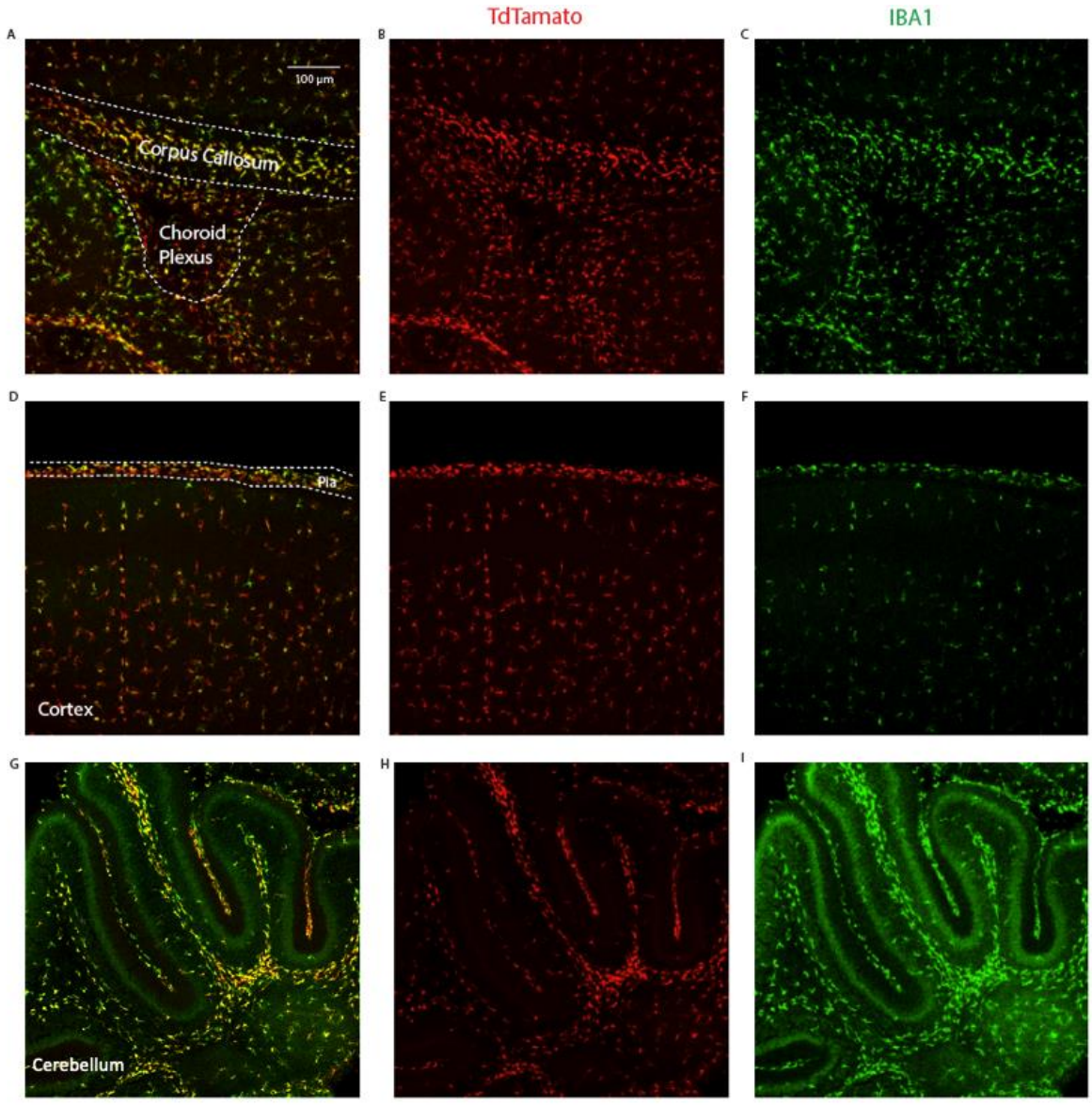
To evaluate the specificity of recombination, we also examined recombination in non-microglial cell types in the brain. Using immunohistochemistry in cortical brain sections, we did not detect any tdT expression in NeuN-expressing neurons or Olig2-expressing cells of the oligodendrocyte lineage (***Figure 9***). To further determine the specificity of Fcrls-Cre recombination, we analyzed

a variety of organs including the thymus, spleen, liver, heart, lung, and intestines. In the liver and skin of Fcrls-Cre; Ai14, we found significant recombination (***Figure 10***). In all the other organs (thymus, heart, lung, large and small intestines), we observed small amounts of recombination. In the spleen, we observed that a significant portion of recombination (tdT^+) is $IBA1^-$, indicating potential leakage into other non-macrophage cells, such as lymphocytes at this stage in development. Together, this suggests that the Fcrls-Cre line can target all subsets of microglia efficiently, including the white matter-associated population, with very good specificity.

In parallel with the creation of Fcrls-Cre mice, we opted to validate a second, recently emerged line regarding the possibility of targeting white matter-associated microglia, while avoiding targeting of other cells. Specifically, we chose to examine the Cx3cr1-Cre (Cx3cr1-CreM line from the MMRRC, not to be confused with $Cx3cr1\text{-}Cre^{Jung}$) in terms of their microglial reporter gene activation pattern that was recently shown in adult mice (Zhao et al., 2019).

In CNS, most microglia were tdTomato positive (data not shown). In the large intestine, liver, lung, and spleen of Cx3cr1-Cre;Ai14, we found strong recombination in $F4/80^+$ cells (***Figure 11***). In all the other organs (skin, kidney, heart), we observed some recombination. This suggests that Cx3cr1-Cre also label tissue-resident macrophages in the peripheral organs. Cx3cr1-Cre;Ai14 recombination is almost completely overlapped with $F4/80^+$ macrophages in the liver and lung. In the large intestine and spleen, however, there is only partial overlap between tdT^+ and $F4/80^+$ cells. In the spleen especially, we observed that a significant portion of recombination (TdT^+) is $F4/80^-$, indicating potential leakage into other non-macrophage cells, such as lymphocytes. Together,

these data suggest that while the Cx3cr1-CreM line also targets microglia, it is recombining loxP sites in other myeloid and lymphoid subsets to a large degree.



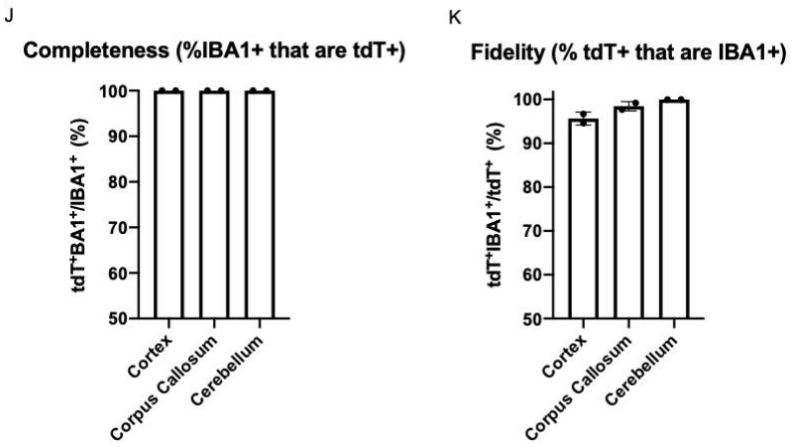


Figure 8 Recombination of Fcrls-Cre in the corpus callosum, choroid plexus, cortex, pia, and cerebellum regions of the brain (T. Kaiser, L. Li)**.** *Representative immunostaining showing tdT and IBA1 expression in the **(A-C)** corpus callosum and choroid plexus, **(D-F)** cortex and pia, **(G-I)** cerebellum of Fcrls-Cre;Ai14 mice. Recombination was seen in IBA1$^+$ macrophages in the choroid plexus and pia. (J-K) Quantification of completeness and fidelity of recombination in the cortex, corpus callosum, and cerebellum.*

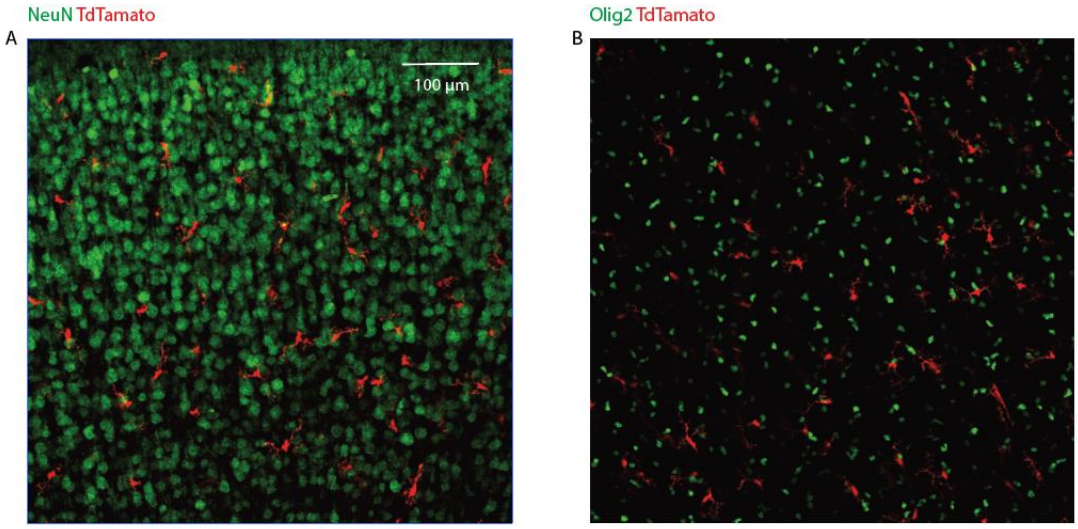


Figure 9 Fcrls-Cre mice do not recombine loxP sites in neurons and oligodendrocytes (T. Kaiser, L. Li)**.** *Representative immunostaining for neurons (A, NeuN, green) and oligodendrocytes (B, Olig2, green). No recombination was seen in NeuN and Olig2 expressing cells.*

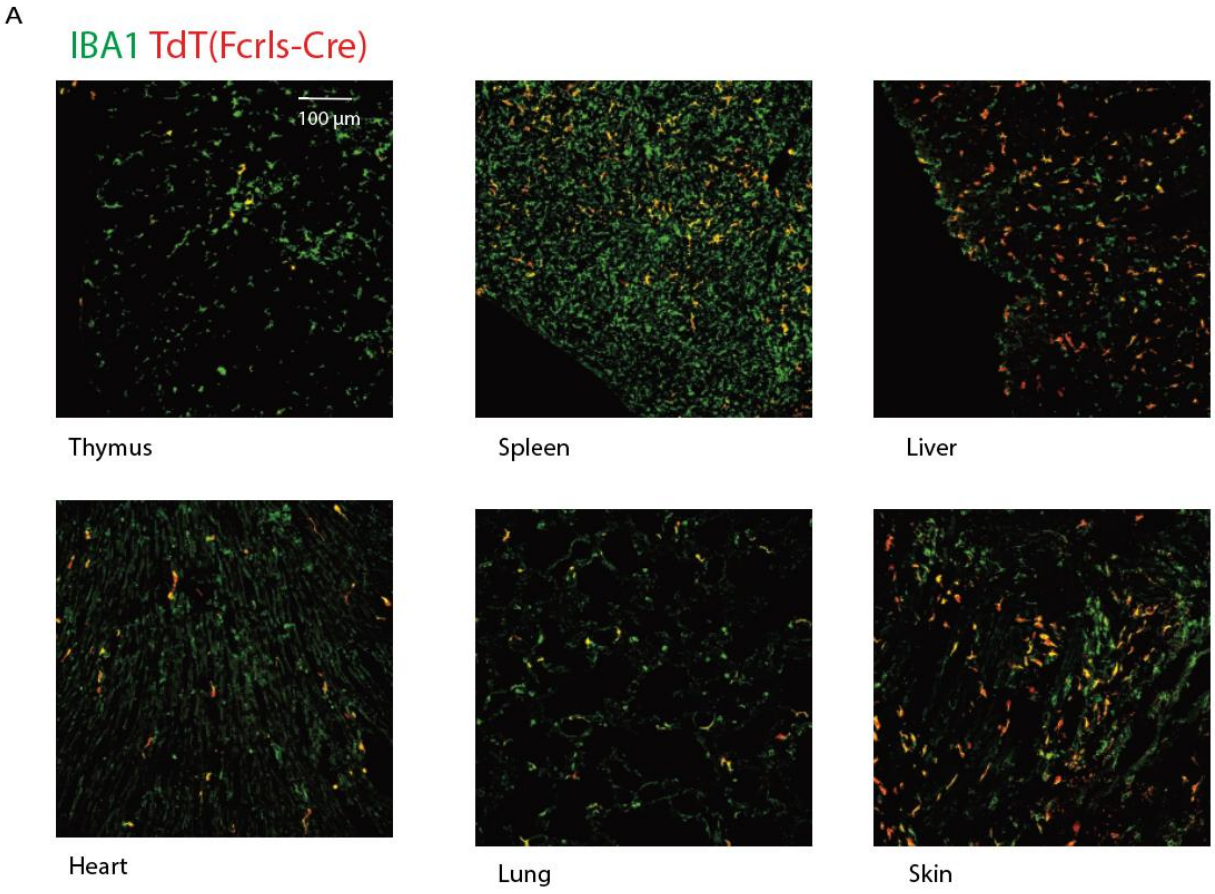


Figure 10 Fcrls-Cre recombine loxP sites in subsets of cells in the thymus, spleen, liver, heart, lung, intestines, and skin of Fcrls-Cre;Ai14 mice (L. Li). **A.** *Representative confocal images showing tdT and IBA1 expression in each organ B. Quantification of recombination in IBA1 macrophages*

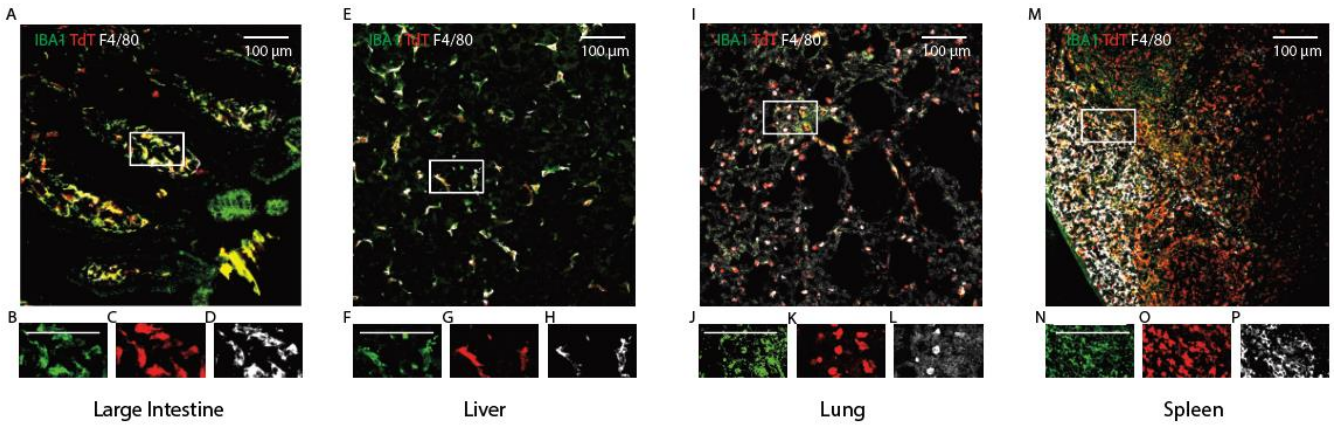


Figure 11 Representative immunofluorescence images of tdT(red)-expressing Iba1+(green) F4/80+ (grey) macrophages in the large intestine, liver, lung, and spleen of adult Cx3cr1-CreM (L. Li)**.**

2.3 Discussion

To expand the tools to target and manipulate microglia, especially in the white matter-associated population that occurs in early development, we generated new transgenic mouse lines: Fcrls-Cre and Fcrls-CreERT2. While we are continuing to investigate the efficiency and specificity of the two mouse lines, preliminary data showed that Fcrls-Cre recombines loxP sites in microglia successfully. Our Fcrls-Cre line achieved 100% recombination efficiency in the cortex, corpus callosum, and cerebellum. Recombination is absent in non-myeloid cells in the CNS. Our preliminary data showed tdT expression in a few IBA1-negative cells, especially near the cortex. This may be because these cells are deep in the tissue and thus less accessible to the IBA1 antibody staining while the tdT fluorescent is more easily detectable due to the endogenous tdT expression. We found recombination in pial and choroid plexus macrophages in the CNS and subsets of macrophages in peripheral organs, which was expected based on previous studies (Hammond et al., 2019; Van Hove et al., 2019). scRNA-seq analyses in these studies show that other brain macrophages also express *Fcrls* at very low levels. Given that Cre/loxP

recombination is an all or none phenomenon, the low-level expression of *Fcrls* in the pial and choroid plexus macrophages is sufficient to recombine floxed alleles and result in tdT expression. Together, this indicates that the Fcrls-Cre line provides a valuable tool to target all subsets of microglia efficiently, including the white matter-associated population in early postnatal development with very good specificity.

There are several existing transgenic mouse models to target microglia *in vivo*. They used different promoters including Cx3cr1, P2ry12, Tmem119, Hexb, and Sall1. The mouse line using the Cx3cr1 promoter by Jung et al. was reported to have significant leakage into neurons (Haimon et al., 2018). The P2ry12-CreER line is reasonably microglia specific, but a subset of dural and choroid plexus macrophages are also recombined in addition to microglia (McKinsey et al., 2020). Sall1-based microglial recombinase lines, although reported to exclude CAMs, target other CNS cells including astrocytes and oligodendrocytes (Buttgereit et al., 2016). Tmem119-based lines were recently generated, targeting microglia, but *Tmem119* is relatively downregulated compared to other microglia signature genes in some microglia subsets in development (Hammond et al., 2019; T. Kaiser & Feng, 2019). A very recent targeting approach using Hexb, a stably expressed microglia gene during both homeostasis and disease, showed $Hexb^{CreERT2}$ system efficiently targets microglia while avoiding perivascular and subdural macrophages (Masuda et al., 2020). However, based on scRNA-seq data, Hexb is highly expressed in CNS choroid plexus macrophages and monocytes (https://www.brainimmuneatlas.org/tsne-cp.php (Van Hove et al., 2019)). Another important study came out very recently showing that a split Cre system: $Sall1^{ncre}$: $Cx3cr1^{ccre}$ mice can exclusively target parenchymal microglia, although it still lacks the ability to achieve recombination in all subsets of microglia in early development (Kim et al., 2021).

To further determine the specificity of Fcrls-Cre, we need to examine recombination in CAMs using specific CAM markers, especially for perivascular macrophages since they are not easily detectable by anatomical locations. CD206, a marker for perivascular and leptomeningeal macrophages can be used for immunostaining to see if recombination is found in $CD206^{+}$ cells. In addition to immunofluorescence analysis, flow cytometry would be required to assess the recombination of Fcrls-Cre in monocytes and lymphocytes of the peripheral blood. These experiments are currently underway.

Thanks to rapid advances in microglia research, several reporter mice for studying microglia *in vivo* are available. These lines provide valuable resources to investigate microglia, although they all have their limitations. *P2ry12, Cx3cr1, and Tmem119* were expressed at very low levels or not at all in white matter-associated microglia from the developing brain (Hammond et al., 2019). For future research toward more specific lines, the Fcrls locus could perhaps be leveraged in combination with another locus such as Sall1 that can exclude CAMs in a split Cre system. Fcrls-Cre:Sall1-Cre may provide a novel tool to not only exclusively target microglia but also target all subsets of microglia with high efficiency.

3. Investigating the role of IgG-FcRγ-mediated signaling in the development of myelin

3.1 Introduction

Antibodies, also known as immunoglobulins (Ig), are composed of two heavy and two light chains, each of which contains an NH2-terminal that is variable and required for antigen binding and a COOH-terminal (Fridman, 1991). The COOH-terminal domains of Ig heavy chains form the Fc region, which is constant in all antibody molecules of a given isotype. The Fc fragment is responsible for triggering cellular activities through its interaction with specific receptors (FcRs). Immunoglobulin G (IgG) is one of the five immunoglobulin isotypes and also the most abundant in human serum (Vidarsson et al., 2014). IgG binds to Fc receptors for IgG (FcγR). FcγRs are important receptors known to have important functions in various immune responses (Takai et al., 1994). There are four different classes of FcγRs and they can be distinguished by two criteria: their affinity for the antibody and whether they are activating or inhibiting cell responses (Chauhan et al., 2017). Mice and humans have one high-affinity receptor, FcγRI and all other FcγRs have low to medium affinity for the antibody Fc fragment. In terms of the signals triggered, there is only one inhibitory FcγR (FcγRIIB) and the other three FcγRs (FcγRI, FcγRIII, and FcγRIV) are activating in mice (***Figure 12***). All of these activating Fc receptors share the same γ chain (FcRγ, not to be confused with FcγR) for their signal transduction (M. J. Wilson et al., 2000).

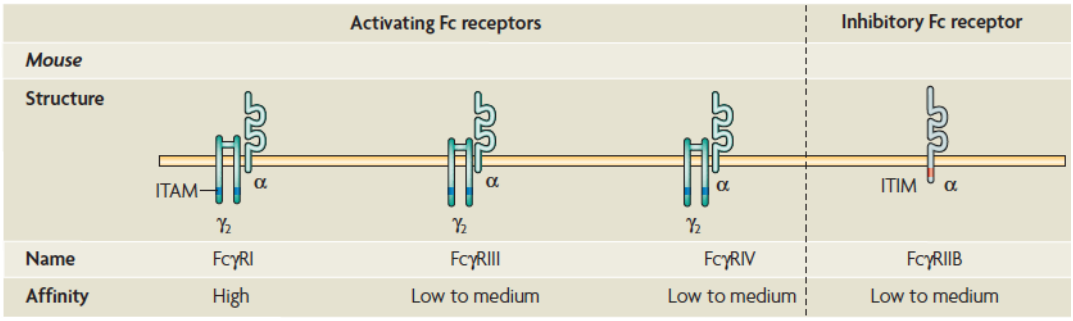


Figure 12 Schematic of the family of Fc receptors for IgG in the mouse (Adapted from Nimmerjahn & Ravetch, 2008). *There are four types for mice: one high-affinity receptor, FcγRI and all other FcRs have low to medium affinity for the Fc fragment. All the activating FcRs (FcγRI, FcγRIII, and FcγRIV) consist of a ligand-binding α chain and a signal-transducing γ chain dimer, which carries immunoreceptor tyrosine-based activating motifs (ITAMs).*

FcRγ is a homodimer of two chains linked via a disulfide bond and each chain contains one ITAM. FcRγ is not involved in ligand binding of the receptor, however. After crosslinking, kinases of the SRC family phosphorylate the tyrosine residues of the FcRγ ITAM that provide a docking site for kinases of the SYK family (***Figure 13)***. This triggers several downstream signaling events and leads to increased intracellular calcium levels. Besides calcium-dependent pathways, the RAS-RAF-MAPK (mitogen-activated protein kinase) pathway is also activated. These signals can activate the immune cell and lead to ADCC (antibody-dependent cellular cytotoxicity), phagocytosis, oxidative burst, and cytokine release (Nimmerjahn & Ravetch, 2008).

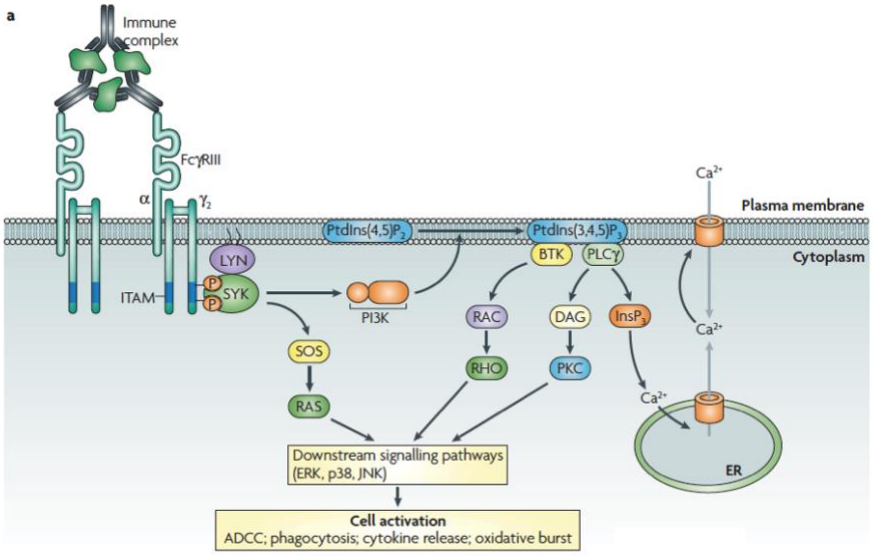


Figure 13 Signaling pathways triggered by activating FcγRs (Adapted from Nimmerjahn & Ravetch, 2008). *Crosslinking of activating Fc receptors for IgG (FcγRs) by immune complexes induces the phosphorylation of receptor-associated γ-chains by SRC kinase family members. This generates SRC homology 2 (SH2) docking sites for SYK, which in turn activates a number of other signal-transduction molecules such as phosphoinositide 3-kinase (PI3K) and son of sevenless homolog (SOS). The generation of phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P3) recruits Bruton's tyrosine kinase (BTK) and phospholipase Cγ (PLCγ), which leads to activation of downstream kinases and the release of calcium from the endoplasmic reticulum (ER).*

An earlier study by Nakahara et al. suggested that signaling via FcRγ may induce oligodendrocyte precursor cell differentiation (Nakahara et al., 2003). Their evidence showed FcRγ is expressed in oligodendrocytes and their precursor cells (OPCs) both *in vitro* and *in vivo*. However, it is important to note that Nakahara and colleagues attributed the expression to OPCs based on relatively low-resolution immunohistochemistry. Since the Nakahara study in 2003, cell profiling technologies including cell-type specific RNA sequencing, single-cell RNA sequencing, and high-resolution immunostaining have substantially improved resolution in analyses. Recent studies using bulk RNA sequencing from sorted or panned cell types, as well as single-cell analysis have shed new light on the cell-type-specific expression of FcRγ, suggesting that FcRγ is almost exclusively expressed in microglia, not oligodendrocytes (***Figure 14***, Marques et al., 2016; Saunders et al., 2018; Y. Zhang et al., 2014).

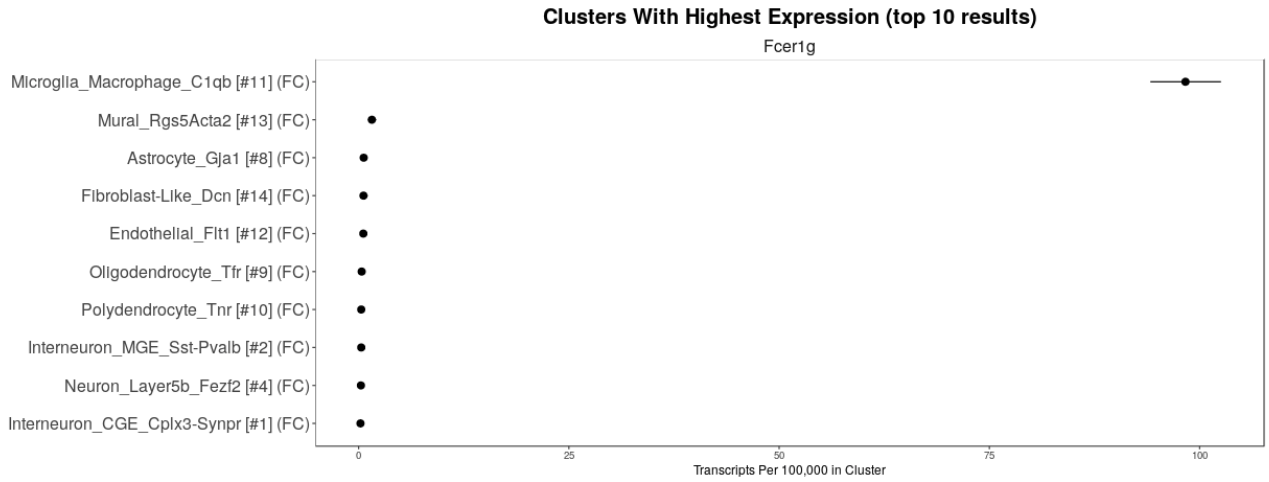


Figure 14 Fcer1g expression profile in the cells of the adult mouse brain in the frontal cortex. *Adapted from "Dropviz"*

To show the functional relevance of FcRγ signaling for myelination, Nakahara and colleagues stimulated OPCs in mixed cultures with anti-FcRγ antibody or IgG for 24h. OPCs demonstrated significant morphological differentiation and acquired well-developed processes (***Figure 15*** Ab & Ac). Interestingly, OPCs in mixed cultures from FcRγ-deficient mice did not differentiate under either anti-FcRγ antibody or IgG stimulation (***Figure 15*** Ae and Af). Their finding that both IgG and anti-FcRγ antibody-induced differentiation of OPC is consistent with our current hypothesis that IgG binds to FcγR and that signal transduction occurs via FcRγ to induce myelination (Nimmerjahn & Ravetch, 2008), possibly through microglia present in the mixed cultures.

In addition, Nakahara and colleagues analyzed myelin basic protein (MBP) expression following stimulation by anti-FcRγ antibody and by IgG and found increased MBP expression (***Figure 15*** Ba). Mice deficient in FcRγ are hypomyelinated and have significantly reduced MBP content. Together, they hypothesized that FcRγ crosslinking on OPCs by IgG stimulates Fyn signaling and induces the rapid morphological differentiation with upregulation of MBP.

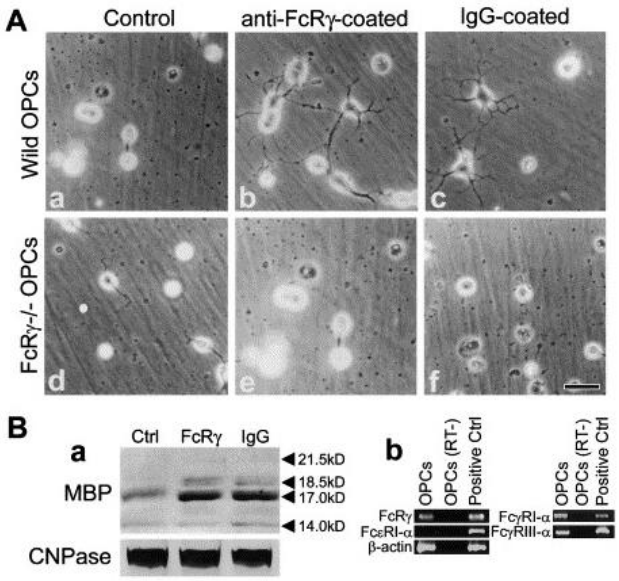


Figure 15 Signaling via FcRγ induces OPC differentiation, adapted from (Nakahara et al., 2003). *(A) Following stimulation with anti-FcRγ antibody for 24hr, OPCs demonstrate dramatic morphological differentiation, acquiring well-developed processes (Ab). Similar differentiation is also observed when IgG was substituted for the antibody (Ac). Neither anti-FcRγ antibody (Ae) nor IgG (Af) differentiates OPCs derived from FcRγ-deficient mice. Control, without the antibody or IgG (Aa and Ad). (Ba) Western blot analysis of MBP expression following stimulation. Cells in (A) were lysed and analyzed. MBP is upregulated in OPCs following stimulation by anti-FcRγ antibodies (lane 2) or by IgG (lane 3), compared to controls (lane 1). (Bb) RT-PCR analysis reveals that the α chains of FcγRI/III (Fc receptors for IgG), but not of FcεRI (Fc receptor for IgE), are detectable in bulk RNA extracts from mixed OPC cultures.*

Interestingly, during embryonic and early postnatal development, *Fcer1g* is upregulated based on recent scRNA-seq data, consistent with the hypothesis that *Fcer1g* may play important roles in myelin development (***Figure 16***). Taken together, the gamma chain of the Fc receptors (FcRγ, encoded by *Fcer1g)* may be involved in myelination and it may contribute to oligodendrocyte maturation through signaling triggered by IgG.

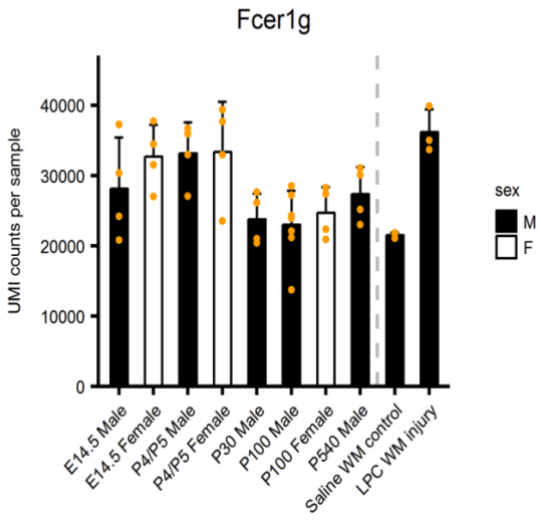


Figure 16 Fcer1g is upregulated in early developmental microglia (Adapted from Hammond et al., 2019) *UMI counts per sample showing expression levels of Fcer1g at different time points and states.*

The previous study (Nakahara et al., 2003) suggests that IgG triggers the activation of FcRγ, inducing differentiation of OPC. This implies that IgG may enter the CNS through yet unknown mechanisms and play a role in myelinogenesis. We also know that IgG is the only class of antibody that is actively transported from mother to the fetus and this specific transport of IgG is mediated through the neonatal Fc receptor: FcRn (Roopenian & Akilesh, 2007). In rodents and humans, the neonatal Fc receptor for IgG (FcRn) binds to maternal IgG in an acidic environment, transcytoses it across a polarized epithelial cell barrier, and releases it to the neonatal blood (in rodents) or fetal circulation (in humans) (***Figure 17***). If these receptors can transport IgG from a mother to the fetus, they may also participate in the transport of IgG across the blood-brain barrier. There is evidence showing that FcRn is indeed expressed at the blood-brain barrier and exports IgG from the CNS into the circulation (Schlachetzki et al., 2002; Yun Zhang & Pardridge, 2001). However, it is unclear if these FcRn also plays a role in transporting IgG into the CNS.

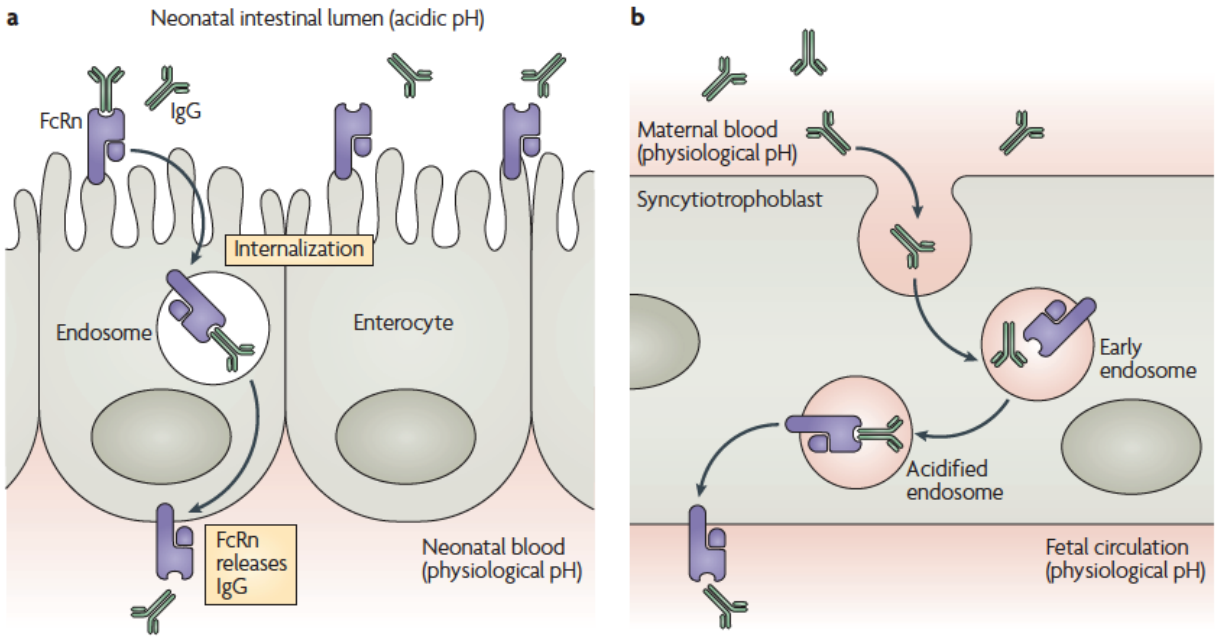


Figure 17 FcRn mediates the perinatal transfer of IgG (Adapted from Roopenian & Akilesh, 2007). *a. In rodents, FcRn is expressed on the cell surface of enterocytes that is covered with microvilli (brush border). Shortly after birth, rodent pups ingest maternal milk containing IgG, which binds FcRn on the brush border in the acidic milieu of the duodenum. After binding, FcRn transcytoses IgG and releases it at neutral pH on the neonatal side. b. In humans, the bulk of materno fetal IgG transfer occurs across the syncytiotrophoblast of the placenta. FcRn is expressed in the internal vesicles of the syncytiotrophoblast.*

3.2 Results

3.2.1 Fcer1g is specifically expressed in microglia

To confirm the expression profile of *Fcer1g* in the brain, we conducted smFISH for *Fcer1g*, *Fcrls*, and *Olig2*. Our preliminary data (T. Kaiser, L. Li, G. Feng, unpublished) showed that all of the *Fcer1g* positive cells co-localize with the microglial marker Fcrls but not oligodendrocytes marker Olig2. This concludes that Fcer1g RNA is specifically expressed by microglia (***Figure 18***).

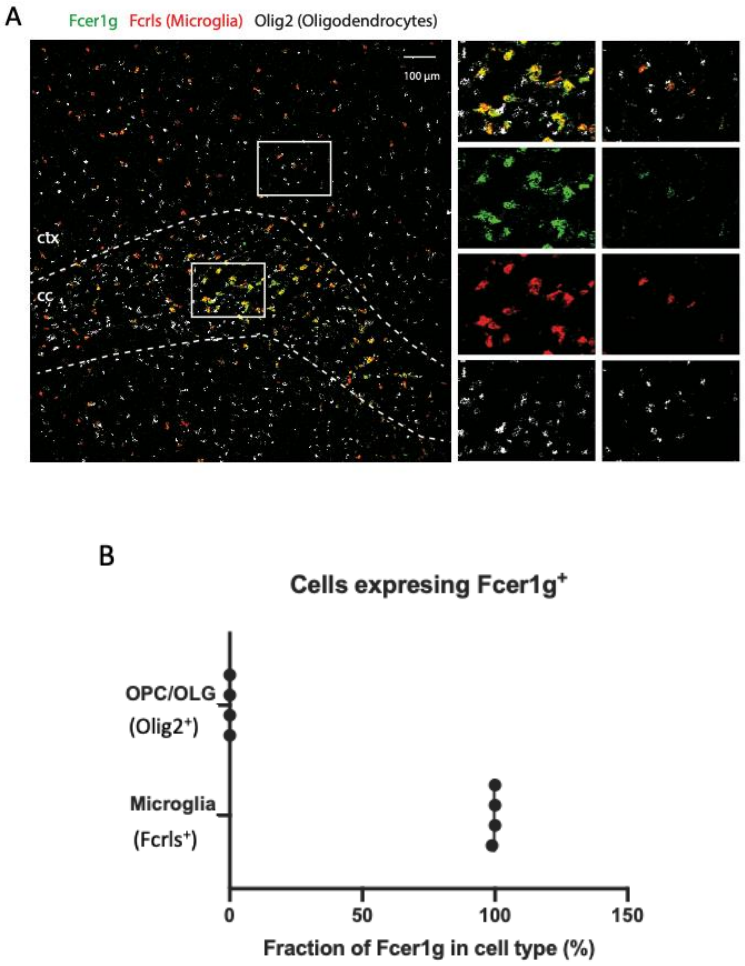


Figure 18 Fcer1g is expressed in microglia but not oligodendrocytes (T. Kaiser, L. Li). *A. Representative confocal microscopic images for oligodendrocytes (Olig2, gray), microglia (Fcrls, red), and Fcer1g$^+$ cells (green). B. Quantification showing the type of cells expressing Fcer1g.*

3.2.2 FcγR ligand IgG occurs in the developmental brain

To determine if FcγR ligand IgG occurs in the early developmental brain, we sacrificed mice at different early developmental time points and conducted western blot and immunohistochemistry to analyze IgG presence and its spatial location. Western blot analysis shows that IgG is found in the developing brain, peaking at P7 followed by a gradual reduction (***Figure 19*** *A*). This result suggests that although IgG amount decreases after P7, IgG is present in the brain throughout the neonatal period with a peak around P7, which is a key time in OPC differentiation. To investigate

the localization of IgG in the developing brain, we performed immunohistochemistry for IgG in mice from WT dams and found that IgG co-localized with IBA1^{+} microglia. In contrast, we did not detect IgG in pups from RAG2$^{-/-}$ dams that lack mature lymphocytes, indicating the specificity of the staining. This suggests that IgG in the developing brain comes from the dam. Together, these preliminary data show that IgG occurs in the developing brain, where it specifically localizes to microglia (T.Kaiser, G.Feng, unpublished).

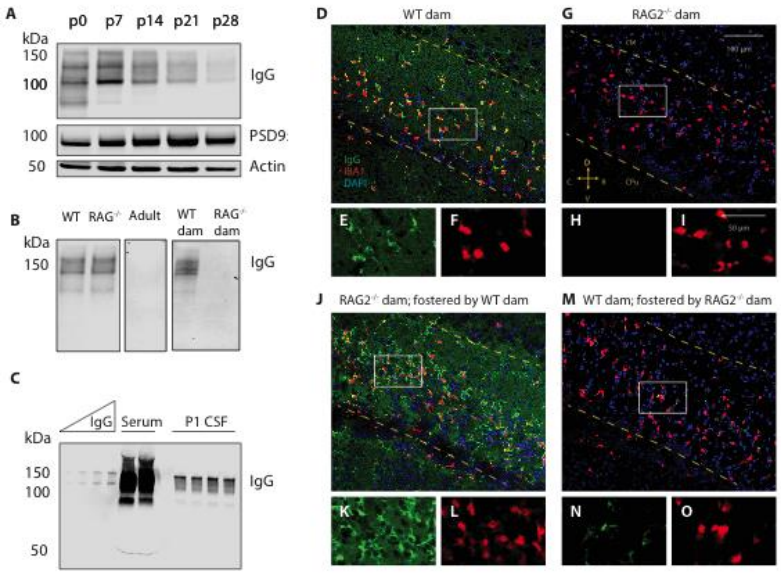


Figure 19 FcγR ligand IgG occurs in the developing brain (T. Kaiser).

3.2.3 Differential expression of key genes in Fcgrt knock out mice

To investigate the mechanisms through which IgG may regulate microglial function and thus affect the development of myelin, we conducted bulk RNA sequencing of sorted microglia. As a complementary model to RAG-deficient mice that allows us to study litter mates, Fcgrt (FcRn protein) knock-out mice were obtained, which lack IgG in the brain due to an inability to transport IgG across the placenta and the gut epithelium (data not shown). After CD11b^{+}CD45lo/int microglia were isolated using FACS, RNA sequencing of sorted microglia was conducted.

Preliminary data shows that *Spp1, Gpnmb, Lgals,* and *Igf1* are the top downregulated genes in the *Fcgrt* knockout mice (***Figure 20***). This suggests two possibilities: there could be fewer Spp1^{+} microglia amongst all microglia in the KO mice or it could mean lower expression of these genes.

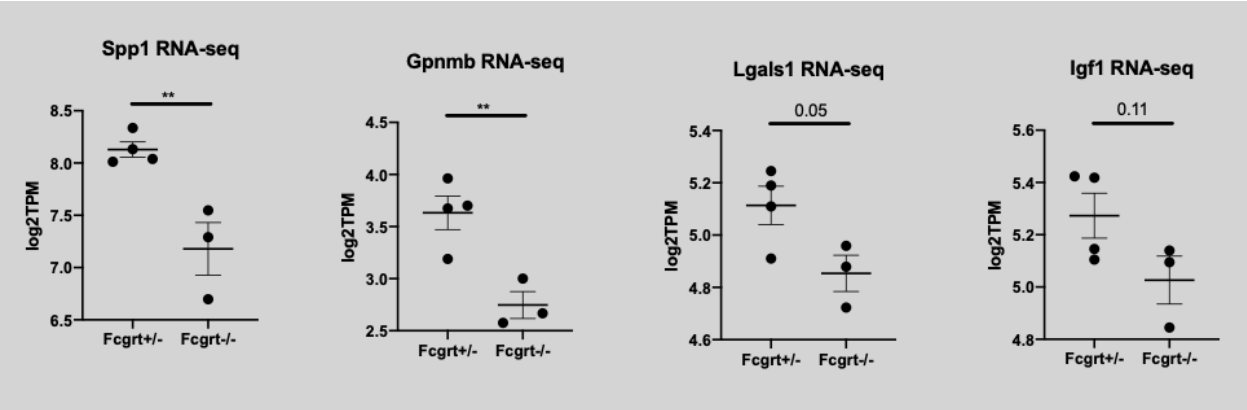


Figure 20 Spp1, Gpnmb, Lgals, and Igf1 are the top downregulated genes in Fcgrt$^{-/-}$ mice. (T. Kaiser)

We then used fluorescent in-situ hybridization and immunohistochemistry to examine whether *Spp1, Gpnmb* are involved in IgG-FcRγ signaling to mediate the regulation of myelination. We performed smFISH for *Spp1* and *Gpnmb* and indeed we found that Spp1^{+} and Gpnmb^{+} cells localize with microglia and reside only in the axon tracts of the corpus callosum in the forebrain and cerebellum (***Figure 21*** & ***Figure 22***). To confirm the RNA sequencing data, we compared the number of Spp1^{+} and Gpnmb^{+} cells between Fcgrt knock out mice and control at P7 by smFISH. Consistent with the RNA-seq data, the results show a significant reduction in Spp1^{+} and Gpnmb^{+} cells at P7 in the Fcgrt knock out mice compared to control mice (***Figure 23***).

To examine the expression level of the white-matter-associated microglia genes at later time points, we also conducted smFISH for Spp1 and Gpnmb at P9 and found that cells expressing these genes were much scarcer in the corpus callosum of wild type mice at this time point (***Figure 24***). These

data, together, demonstrate the potential differential regulation of *Spp1* & *Gpnmb* spatially and temporally by microglia. Their reduced expression in the Fcgrt knock out mice implicates IgG-FcRγ-mediated signaling in the biology of this particular subset of microglia and the subset's potential role in myelin development.

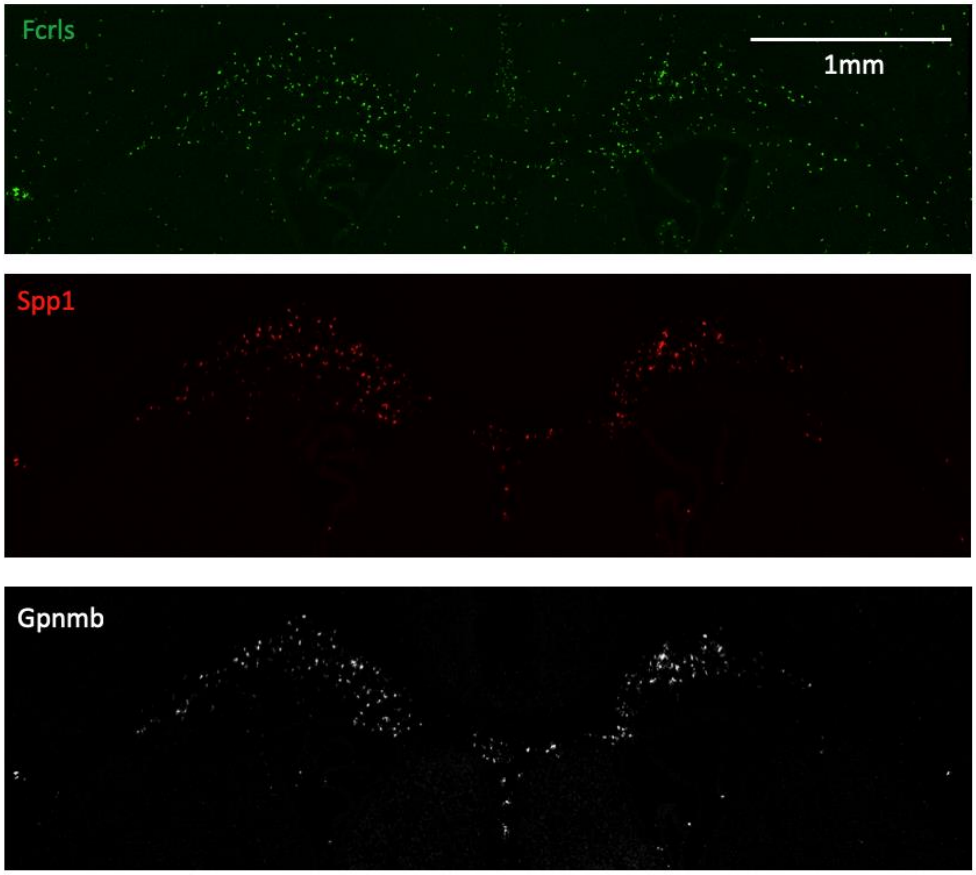


Figure 21 Spp1 and Gpnmb are specifically located in the subcortical axon tracts of the corpus callosum (L. Li). *Confocal images of the P7 axon tracts of the corpus callosum stained by smFISH.*

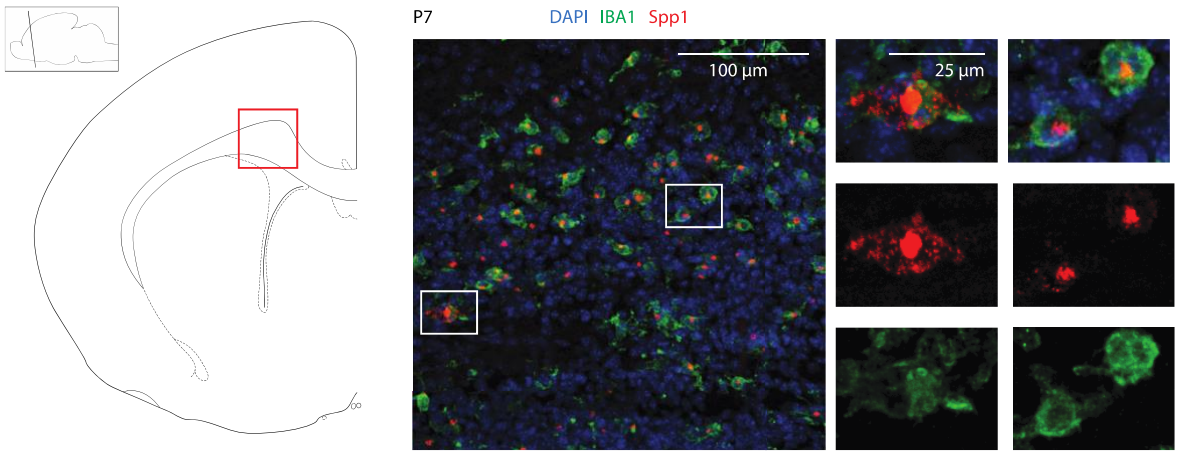


Figure 22 Spp1 is expressed specifically in microglia (T. Kaiser, L. Li)**.** *High-magnification confocal image of the P7 corpus callosum stained by immunofluorescence with probes DAPI, IBA1, and Spp1*

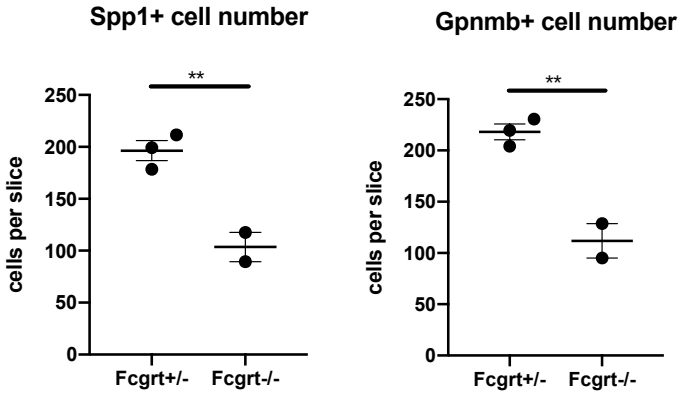


Figure 23 Fcgrt knockout mice (Fcgrt$^{-/-}$) show a significant reduction in Spp1^{+} and Gpnmb^{+} cells by smFISH (T. Kaiser, L. Li)**.** *Quantification of the number of Spp1^{+} and Gpnmb^{+} cells per slice in the control Fcgrt+/- and Fcgrt knock out (Fcgrt$^{-/-}$) mice at P7.*

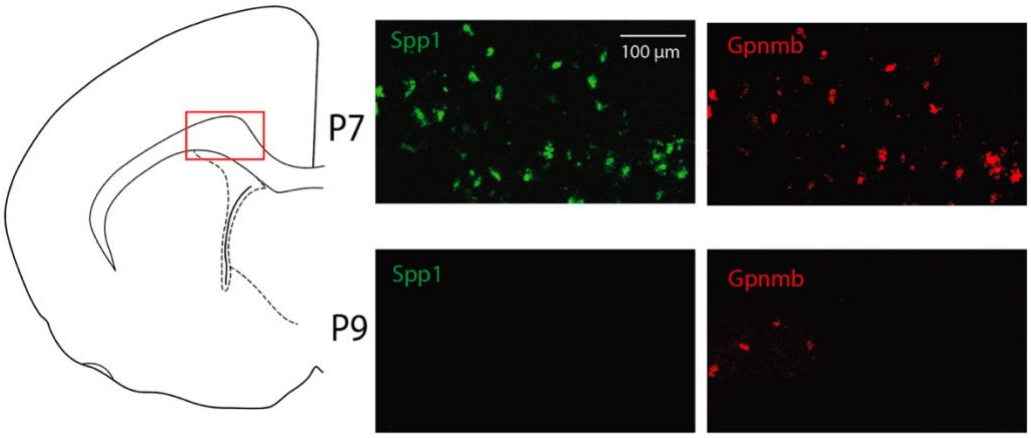


Figure 24 Spp1 & Gpnmb are no longer upregulated at P9 (L. Li). *Confocal images of the P7 and P9 Spp1$^+$ and Gpnmb$^+$ cells in the axon tracts of the corpus callosum of wild type stained by smFISH.*

3.2.4 Selective deletion of Fcer1g in microglia

To determine whether myelin deficiency in FcRγ deficient mice is indeed caused by microglia specifically, we sought strategies to limit *Fcer1g* deletion to microglia. Concurrently with our efforts to develop a new suitable Cre line targeting microglia specifically (Fcrls-Cre), we evaluated additional existing Cre-lines that might target microglia in the early postnatal stage. There are several mouse lines available for Cre-expression that use microglial signature genes Tie2, CX3cr1, P2ry12, and Tmem119 (T. Kaiser & Feng, 2019; McKinsey et al., 2020; Zhao et al., 2019). We started with Tie2-Cre because Tie2, although expressed in endothelial cells, is also highly expressed by yolk sac-derived microglia precursors (Gomez Perdiguero et al., 2015). Hence, we consider this line suitable to target microglia in the early postnatal stage. To confirm this, we crossed Tie2-Cre mice to the Ai-14 reporter line, which expresses tdTomato in the presence of Cre. Confocal microscopic images showed that Tie2 is indeed expressed in all Fcer1g$^+$ microglia in addition to endothelial cells (***Figure 25***).

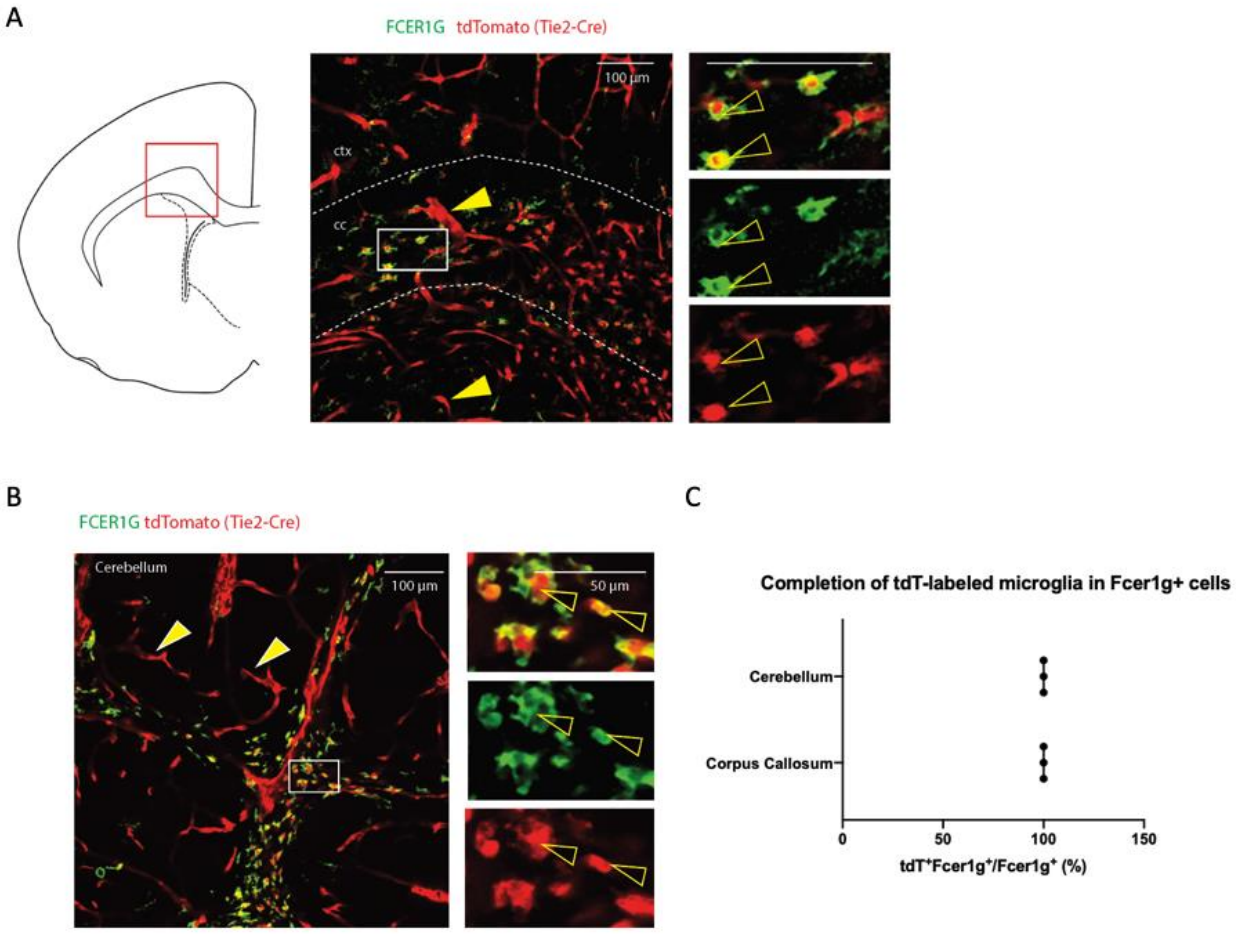


Figure 25 Tie2-Cre targets microglia along with endothelial cells in the corpus callosum and cerebellum (L. Li). *A and B. Representative confocal images showing tdTomato (Tie2) expression (red) in Fcer1g$^+$ microglia (green) in the axon tracts of corpus callosum (A) and cerebellum (B). The open arrowheads indicate tdT-labeled Fcer1g$^+$ microglia. Closed arrowheads indicate tdT$^+$Fcer1g$^-$ endothelial cells. Abbreviations: ctx, cortex; cc, corpus callosum. C. Quantification of completion of tdT-labeled microglia in Fcer1g$^+$ cells.*

3.3 Discussion

The previous study by Nakahara shows that FcRγ (encoded by *Fcer1g*), the common gamma subunit of Fc receptors, is expressed by cells of the oligodendrocyte lineage both *in vitro* and *in vivo* (Nakahara et al., 2003). However, our results demonstrate that FcRγ is specifically expressed in microglia, not cells of the oligodendrocyte lineage, which is consistent with the recent scRNA-

seq data. Interestingly, smFISH data showed that *Fcer1g* expression is highly concentrated in the corpus callosum compared to other regions. FcRγ is reported to promote OPC differentiation and deficiency in FcRγ resulted in decreases in myelin basic protein and hypomyelination (Nakahara et al., 2003). Together, these data substantiated our hypothesis that microglial FcRγ is required for the development of myelin.

In Nakahara's experiments, they showed that the α chains of FcγRI/III (Fc receptors specific for IgG) but not of FcεRI (Fc receptor for IgE) are expressed in OPCs from mixed cultures (***Figure 15*** Bb), raising the possibility that IgG can be the extracellular triggering molecule that activates FcRγ through the α chains. Our preliminary data do show that IgGs are present in the developing brain and bind specifically to microglia. Through the comparison of WT dam and $RAG2^{-/-}$ dam and cross-fostering experiments, we concluded that IgG comes through both cross-placental transfer and the maternal milk. It has been known that the receptor responsible for transferring maternal IgG to rodent pups is the neonatal Fc receptor (FcRn). FcRn, encoded by Fcgrt, transcytoses IgG and releases it into the neonatal blood. FcRn is also known to be expressed at the blood-brain barrier and believed to mediate the reverse transcytosis of IgG in the brain to blood after direct intracerebral injection of IgG (Schlachetzki et al., 2002), but the mechanism by which IgG crosses the BBB and enters the brain is still unclear. Nevertheless, we did find IgG in the developing brain, peaking at P7 and gradually disappearing after that. Mice with $RAG2^{-/-}$ dam, however, did not have detectable levels of IgG in the brain, suggesting that IgG comes from maternal milk, transcytoses across the epithelial cell barrier of enterocytes into the blood, crosses the BBB, and enters the CNS. The result is particularly interesting in that the brain is believed to have immune privileges due to the fact that brain endothelial cells, unlike peripheral endothelial

cells, have tight junctions and also interact with astrocytes and pericytes to form a blood-brain barrier to prevent large molecules, such as IgG from accessing the brain. As such, it is intriguing to see that IgG is able to cross BBB in early development. Although little is known about the mechanism, several possibilities could explain the transcytosis of IgG in development. First, IgGs could enter through circumventricular organs, which line the cavity of the third ventricle and fourth ventricle and are devoid of BBB (Duvernoy & Risold, 2007). Second, FcRn, the only known Fc receptor to be expressed by brain endothelial cells (Villaseñor et al., 2016) could facilitate the transcytosis of IgG from blood to CNS during early development. Additional experiments using specific markers of FcRn would be required to confirm this hypothesis. Alternatively, other unknown receptors could transport IgG across BBB.

It is important to note that FcRγ, the gamma subunit is shared by other activating FcRs such as FcαRI and FcεRI with different ligands (IgM, IgE) than IgG (Ben Mkaddem et al., 2019). Therefore, additional work would be required to confirm that only IgG, but not other immunoglobulins, interact with FcRγ in microglia. In addition, FcγRI, FcγRIII, and FcγRIV (FcγR activating receptors in mouse) all have common gamma chain FcRγ, and identifying which combination of the receptors are expressed in microglia would further elucidate microglial FcR biology.

To probe the mechanism by which IgG-FcRγ mediates myelination, we sorted microglia from Fcgrt knockout mice and conducted RNA-sequencing. The RNA sequencing result is interesting in that the top downregulated genes identified in Fcgrt knock-out mice, *Spp1, Gpnmb, Lgals,1* and *Igf1* are also the signature genes that identify the subset of axon tract-associated microglia during early development (***Figure 20***, Hammond et al., 2019). At P9, smFISH evidence showed that cells

expressing these genes were much scarcer in the corpus callosum (***Figure 24***). Taken together, this indicates that this *Spp1*$^{+}$ microglia population is differentially regulated in a tight developmental window and may play a role in myelin development via IgG-dependent signaling. The evidence that *Spp1* and *Gpnmb* are downregulated in Fcgrt-knock out mice could have a few explanations. It could mean that there are fewer Spp1^{+} microglia amongst all microglia in the KO mice or it could mean lower expression of these genes. scRNA-seq would provide a clearer picture.

The white matter-associated microglia subset characterized by Spp1 and Gpnmb expression may contribute to myelin development through several ways based on the molecular functions of these proteins. *Spp1* encodes the protein osteopontin (OPN) and it has two isoforms, the secretory OPN and the intracellular OPN (sOPN and iOPN) (Cantor & Shinohara, 2009). OPN is a proinflammatory cytokine, which can be secreted from many cells, including activated macrophages, T-lymphocytes, and also microglia in the CNS (Yu et al., 2017). It was found that OPN expression was significantly increased by microglia under stress and OPN increases microglia survival under stress conditions and has an anti-inflammatory effect in moderate inflammatory environments (Rabenstein et al., 2016; K. X. Wang & Denhardt, 2008). It has recently been reported that OPN plays a role in neurodegenerative diseases such as multiple sclerosis (MS) (Chabas, 2001; Chiocchetti et al., 2005) and Alzheimer's disease (AD) (Wirths et al., 2010). Gpnmb is a type I transmembrane glycoprotein that was initially described in a poorly metastatic melanoma cell line (Weterman et al., 1995). Previous *in vitro* studies showed that Gpnmb can function as a negative regulator of T lymphocyte activation (Chung et al., 2007) and of macrophage inflammatory responses (Ripoll et al., 2007). Therefore, it raises the possibility that Gpnmb produced by microglia acts as a mediator on immune effector cells to reduce excessive

proinflammatory responses in the CNS (Huang et al., 2012). The other downregulated gene in the Fcgrt knock out mice, *Lgals1*, encoding galectin 1, has been associated with tempering microglial activation, brain inflammation, and neurodegeneration (Starossom et al., 2012). Interestingly, it has been reported that Lgals1$^{-/-}$ mice have significantly decreased myelinated axons, and their myelin was more loosely wrapped around axons than in wild-type mice (Rinaldi et al., 2016).

The common function of *Spp1, Gpnmb,* and *Lgals1* that they negatively regulate microglia's proinflammatory responses suggests they may play a protective role in the development of myelin if microglia indeed phagocytose oligodendrocyte or myelin sheath. In addition, the group of early postnatal microglia that exhibits high expression levels of *Spp1* and *Gpnmb* in the corpus callosum and cerebellar white-matter regions have recently been shown to be upregulated in degenerative disease-associated microglia (DAM) as well (Q. Li et al., 2019). In their study, Li and colleagues discovered that this group of microglia specifically expressed CLEC7A protein, which labels microglia surrounding amyloid plaques in mouse models of Alzheimer's disease (AD). Given the similarities between white matter-associated early postnatal microglia and DAM, together with the knowledge that DAM is phagocytic and involved in phagocytosis of amyloid-beta (Aβ) in AD models (Keren-Shaul et al., 2017), there is the possibility that this group of white matter-associated microglia secrets Spp1, Gpnmb and Lgals1 to prevent excessive phagocytosis of oligodendrocytes for proper myelination in early development and this may be mediated through IgG-FcRγ signaling.

3.4 Methods

In situ hybridization

Single molecule fluorescence in situ hybridization (smFISH) was performed using RNAscope Fluorescent Multiplex Kit (Advanced Cell Diagnostics, ACDBio). Isoflurane-anesthetized mice

were decapitated, their brains harvested, and flash frozen on dry ice. Brains were stored at −80 °C. Prior to sectioning, brains were equilibrated to −16 °C in a cryostat for 30 min. Brains were coronally sectioned at 16 μm with cryostat and thaw-mounted onto Superfrost Plus slides (25 x 75 mm, Fisherbrand). Sections from a single brain were serially thaw-mounted onto 5 slides covering the corpus callosum (anterior-posterior distance). Slides were air-dried for 2 hours at room temperature prior to storage at −80 °C. smFISH probes for all genes examined Fcrls, Spp1 and Gpnmb were obtained from ACDBio. Slides were counterstained for the nuclear marker DAPI using ProLong Diamond Antifade mounting medium with DAPI (ThermosFisher).

Immunohistochemistry

Mice were anesthetized and perfused with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (PFA) in PBS. Early postnatal mice were perfused with PBS and PFA. Brains were surgically removed and postfixed in the fixative at 4°C for 24 h. Fixed brains were washed once in PBS and sliced into coronal slices using a Leica VT1000S. Slices were washed three times in PBS, and subject to incubation in blocking solution (5% normal goat serum, 2% bovine serum albumin, 0.2% TX100 in PBS). Blocked sections were incubated with primary antibodies for IBA1 (1:500; Synaptic Systems, 234006), GFP (1:1000, Invitrogen, A11122; 1:500, Aves Labs, GFP-1020), Olig2 (1:1000; Millipore, AB9610), NeuN (1:1000; Millipore, MAB377), GFAP (1:1000; Sigma-Aldrich, G9269) for 24 h at 4°C. Primary antibody incubation was followed by three washes in PBS and incubation with species-matched and Alexa fluorophore-conjugated secondary antibodies raised in goat (Invitrogen; 1:1000) for 2 h. DAPI (1:10.000) was included in a washing step or secondary antibody incubation. Slices were washed three times in PBS and mounted and coverslipped using VECTASHIELD H-1000 mounting medium. For imaging, slides were scanned

on an Olympus FluoView FV1000 fixed stage confocal microscope (high-power magnifications) or Olympus BX61 epifluorescence microscope (sagittal section montage) using built-in software. For microglia morphology analysis, *z*-stacks were acquired at 10X and 20X magnification.

Genetic Analysis of Fcrls-CreERT2 mice

Founder mice were genetically analyzed by amplifying sequences spanning the entire knocked-in sequence. Two amplicons were designed that would overlap in order to cover the entire sequence. The first amplicon spanning about 2.7 kb of the sequence was generated using the forward primer 5' of LHA (5'-AGGTGATGGTGCCTAAAGAC-3') and the reverse primer (5'-GGTCTGGTAGGATCATACTCG-3'). The second amplicon approximately 3.2kb was generated with the forward primer (5'- GATCATGCAAGCTGGTGG -3') and the reverse primer 3' of RHA (5' – CCATCTAGAAGGAGACGGC-3'). Both amplicons were purified using Zymoclean Gel DNA Recovery kit and then sequenced using Sanger sequencing with primers covering the entire sequence.

Quantification of RNAscope

Images from each channel are processed in Ilastik that uses machine learning algorithms to separate signal from background. Processed images are exported as binary masks. A CellProfiler pipeline is designed to process the Ilastik output images. Positive signals are identified and counted by the pipeline.

Statistical analysis

Quantitative data from smFISH and immunofluorescence were analyzed using GraphPad prism.

4. **Conclusions and future work**

In this thesis, we first characterize a novel constitutive Cre mouse line: Fcrls-Cre and our preliminary data show that Fcrls-Cre achieves recombination in all subsets of microglia, including the white matter-associated subset. More cohort studies, however, are required to confirm this. Fcrls-Cre is one of the few Cre-lines that can target microglia with such high efficiency across all subsets. Going forward, new recombinase lines and targeting strategies to target microglia with high efficiency and specificity will be of paramount importance to further our understanding of microglial function in development, homeostasis, and disease.

Second, we investigate the role of microglia, specifically the white matter-associated population in the development of myelin. These white matter-associated microglia are characterized by high expression levels of *Spp1*. *Gpnmb* and *Lgals1*. Our preliminary results show that IgGs are present in the early developmental brain and specifically localize to microglia. In addition, Fcgrt knockout mice ($Fcgrt^{-/-}$) show a significant reduction in $Spp1^{+}$ and $Gpnmb^{+}$ cells. Further analysis, such as scRNA-seq, would be required to elucidate this result. More phenotype studies including assessment of myelin protein levels or morphological analysis are also essential to establish the association between the lack of microglial genes and myelin deficiency. In addition, there are a few future studies that would be of interest to study the contribution of microglia to myelination. First of all, it could be worthwhile to study the functional roles of *Spp1* and *Gpnmb* to determine if they are involved in myelin development or they are mere markers for this white matter-associated microglia. Second, since the signature genes in the white matter-associated microglia are also upregulated in disease-associated microglia that are characterized by their phagocytic

function. As such, phagocytosis studies in early development would provide a clearer picture as to how microglia influence development.

Previous studies show that immune abnormalities at neonatal stages are implicated in neurodevelopmental disorders, including ASD and schizophrenia (Estes & McAllister, 2015). Similarly, myelin abnormalities are also manifested in neurodevelopmental diseases (Graciarena et al., 2019; Uranova et al., 2011). These observations suggest that functions of the immune system and myelin development may be essential to understanding the pathology of neurodevelopmental disorders. Although it remains unclear if abnormal myelin development is associated with immune functions, such as functions mediated by IgGs, it is possible that immune dysfunction leads to myelin deficiency associated with neurodevelopmental disorders. This possibility needs to be further explored in the future.

Author contributions:

L.Li collaborated with T.Kaiser on most of the data generated. Detailed contributions can be seen under each original figure generated.

Appendix

5. Economic, ethical, and policy implications of CRISPR in gene therapy

This part of the thesis is independent of the project described in the first part.

5.1 Introduction

Scientists shaped CRISPR into a precise gene-editing tool not long after microbiologists first discovered this ancient immune system found in archaea and bacteria. Ever since this initial adoption, CRISPR has transformed science in a profound way. The simplicity, flexibility, and affordability of CRISPR have enabled scientists around the world to edit the genetic code of nearly all organisms more efficiently including viruses, bacteria, plants, zebrafish, mice, monkeys, and even humans.

Since its development, CRISPR has been used for many purposes: making new animal models for research, improving the qualities and productivity of crops, and creating new biofuel to meet energy demands. So precise and powerful, CRISPR is also poised to revolutionize the treatment of human genetic diseases. At first glance, this technology offers us an opportunity too promising to refuse, with the potential to correct nature's genetic mistakes and alleviate suffering for those with incurable conditions. On closer inspection, it is anything but simple to decide how far we should go in applying CRISPR, especially pertaining to the human germline.

The promise that CIRSPR can correct genetic mutations raises other societal questions in addition to technical ones. Here, in an effort to inform and frame the discussions, I briefly explore and

summarize the CRISPR technology, its use in the context of gene therapy, and the current clinical trials that use CRISPR, followed by a review of major economic, ethical, and regulatory implications of gene editing.

5.2 Background

5.2.1 Overview of CRISPR/Cas technologies

Bacteria and archaea have an RNA-mediated adaptive defense system called clustered regularly interspaced short palindromic repeats (CRISPR) (Mojica et al., 2005). Remarkably, it is not the repeats, but what is in between these repeats, called spacers that are significant. When a bacteriophage infects a bacterial cell, bacteria capture fragments of viral DNA and integrate these snippets of captured viral code into their own genome for future defense (Barrangou et al., 2007). Each viral fragment, called a spacer is separated by an identical repetitive DNA sequence that is also palindromic and together they form a CRISPR array. The next time a virus attacks the bacteria, this CRISPR array is activated, producing a complementary RNA. The RNA is then processed into individual crRNAs, each crRNA derived from a different virus (Brouns et al., 2008). The RNA is accompanied by a DNA-cutting enzyme called Cas (CRISPR-associated sequence). There are several types of CRISPR system and type II is one of the simplest, featuring an enzyme called Cas9. CrRNA scans the viral DNA for a match and once encountered, Cas9 cuts both strands of the viral DNA.

Cells are armed with multiple molecular pathways to repair double-stranded breaks (DSB) and other mutations in DNA. Two major pathways are called non-homologous end joining (NHEJ) and homology-directed repair (HDR) (***Figure 26***). NHEJ stitches the broken ends of DNA back together but often leads to small insertions or deletions. This is ideal for producing gene knockouts,

where the function of a gene is disrupted by introducing random insertions and deletions. Alternatively, a repair template with homology to the target site can be used to make an error-free edit through HDR. In normal circumstances, the template is the homologous sequence on the sister chromosome. What is ingenious about the adoption of the CRISPR system for research is that we can design the repair template so that it contains the desired DNA sequence flanked by homology arms to be incorporated into the break, leading to a precise edit.

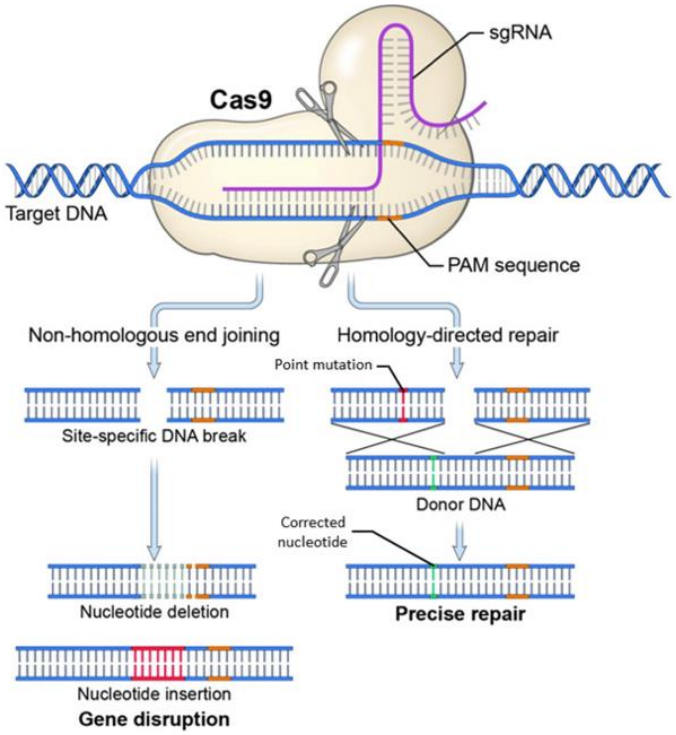


Figure 26 CRISPR-induced repair pathways NHEJ and HDR (Adapted from Guitart et al., 2016). *Upon Cas9-induced DNA DSB, the cell repairs the DSB by either NHEJ or HDR. In NHEJ, random nucleotide insertions and deletions occur as the cell ligates the DNA DSB, often resulting in gene disruption through frameshift. In HDR, the DSB is repaired using an externally supplied homologous DNA as a template for repair. The nucleotide sequence of the donor template is copied into the targeted site, resulting in a directed precise repair.*

Since the discovery of this acquired immune system, initially in archaea, researchers have repurposed the CRISPR system into a precise gene editing tool, not only in bacteria but also adapted to mammalian cells (Cong et al., 2013; Jinek et al., 2012). Instead of a virally-derived

RNA, scientists can program synthetic guide RNAs that allow them to target DNA sequences of their choosing. This is a paradigm shift for gene editing.

Gene editing started before the discovery of CRISPR, with the development of zinc finger nuclease (ZFN) first, followed by transcription activator-like effector nucleases (TALENs). Although these previous generations of tools can induce genome editing, the CRISPR/Cas 9 system offers many advantages over ZFN and TALEN (H. Li et al., 2020). For example, ZFN or TALEN tools require reengineering of the enzyme to fit each target sequence, whereas the nuclease Cas9 in the CRISPR system can be conveniently used in all cases. Moreover, compared to CRISPR/Cas, ZFN and TALEN are much more labor-intensive and more expensive. In addition, CRISPR/Cas offers the possibility of modifying several genomic sites simultaneously (multiplexing). Together, these advantages make the CRISPR/Cas system simpler, cheaper, and more efficient compared to other gene-editing technologies.

Since its early adoption, the CRISPR toolkit has expanded at an astonishing rate. Researchers have discovered new Cas enzymes, engineered new guide RNAs for better specificity, and found ways to target RNA instead of DNA. New generations of editing methods built on the original CRISPR/Cas system also came out recently, with base editing allowing us to target single bases and prime editing promising greater editing flexibility and precision (Anzalone et al., 2019; Komor et al., 2016).

5.2.2 Applications of CRISPR/Cas technologies

The discovery of the CRISPR-Cas system has inspired thousands of new researchers to apply it in a host of settings, from diagnostic platforms that can detect infections such as COVID-19, flu, and Zika, to agriculture and energy applications to grow healthier foods and develop new bioenergy

solutions (Pickar-Oliver & Gersbach, 2019). One of the most important applications is to cure diseases by correcting disease-causing genes in patients. Although gene therapy has been explored for decades, the introduction of the CRISPR-Cas system with its ease and precision has presented new promises as well as challenges. In the next section, I will briefly explore traditional gene therapy approaches followed by CRISPR-based gene therapy.

5.2.3 Overview of gene therapy

Gene therapy is a strategy that modifies genes via disruption, correction, or replacement to provide treatments for diseases caused by genetic mutations (Humbert et al., 2012). Genes can be directly delivered into the cell using physical methods, such as microinjection or electroporation (Dunbar et al., 2018). The genes can also be carried in delivery vehicles, such as viral vectors or lipid nanoparticles (Finer & Glorioso, 2017). There are many ways to classify gene therapy approaches and one of them is whether the vector is administered *in vivo* or *ex vivo* (***Figure 27***). For *in vivo*, the vector is administered directly into the patient's body. For *ex vivo,* cells are removed from the body, treated in the lab, and then readministered.

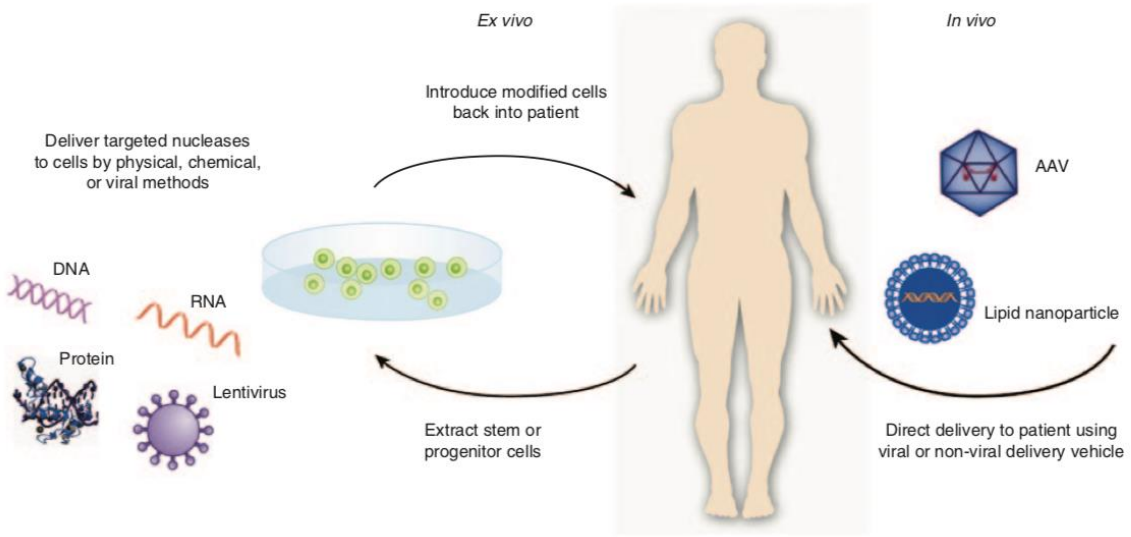


Figure 27 *Ex vivo* and *in vivo* strategies for gene therapy (Adapted from Maeder & Gersbach, 2016).

The first clinical trial that used gene therapy for a therapeutic purpose was approved in 1990 for a monogenic disease - a rare form of severe combined immunodeficiency, caused by a deficiency of the enzyme adenine deaminase (ADA-SCID) (Bordignon et al., 1995). Two girls with ADA-SCID were treated with retroviruses for *ex-vivo* delivery of a wild type adenine deaminase gene to T-lymphocytes. Although the results were not optimal – a follow-up study stated that only about 20 percent of one girls' T cells were producing the ADA enzyme (Blaese et al., 1995), the proof of concept prompted many subsequent gene therapy trials using viral-mediated gene delivery. However, the prospect of gene therapy was met with some major setbacks. Jesse Gelsinger, suffering from a mild form of OTC (ornithine transcarbamylase) deficiency, participated in a clinical trial which delivered a recombinant adenovirus containing a normal copy of the OTC gene to his liver. Unfortunately, Jesse developed a fever and soon passed away. It turns out that the adenovirus vector triggered Jesse's cytokine storm, which led to his death (Raper et al., 2003). This tragedy was soon compounded by another gene therapy trial that resulted in the development of leukemia in several young children induced by oncogenesis from the therapy (Cavazzana-Calvo,

2000). Together, these failures raised considerable concerns about the safety of gene therapy in humans.

In light of Jesse's tragedy, researchers went back to the lab and focused on the basic science of viral vectors and delivery safety (Uddin et al., 2020). Two new candidates emerged as reliable and effective delivery vehicles: adeno-associated viruses (AAV) and lentiviruses. Their discoveries as delivery vehicles sparked a renewed sense of optimism in gene therapy. AAVs are frequently found in humans but not known to cause diseases (Hastie & Samulski, 2015). Today, AAVs and lentivirus are the leading viral vector platforms for *in vivo* and *ex vivo* delivery of gene therapies, respectively. AAV was discovered in the mid-1960s as a contaminant of an adenovirus preparation (Atchison et al., 1965). AAV holds a payload of single-stranded DNA of about 5kb. AAV vectors mostly do not integrate into the host genome, but rather persist with cells as episomal DNA (D. Wang et al., 2019). This means that the DNA payload will get diluted over time if the cells the AAV infects divide, thereby diminishing the treatment effects. As such, AAVs are typically used in non-dividing target cells, such as cells in the eyes, nervous system, and skeletal muscles. However, one of the limitations of AAV is that it can only package a relatively small amount of genetic cargo, posing constraints on the size of the gene-editing machinery it can carry (Wu et al., 2010). Lentivirus is a subtype of retrovirus, so they carry RNA molecules and undergo reverse transcription. The difference in lentiviral vectors, however, is that they can integrate into the host genome (Milone & O'Doherty, 2018). This makes lentivirus best suited for *ex vivo* applications in dividing cells, such as T cells and stem cells.

I will briefly summarize the major gene therapy treatments below, both *ex vivo* and *in vivo*. Although these previous generations of gene therapy do not use CRISPR, they paved the way for later CRISPR-based therapy.

Ex vivo gene therapy

Ex vivo therapy appears to be the safer approach since cells treated in the lab can be subject to strict quality control before being transplanted. Because the *ex vivo* approach requires the removal of diseased cells from the body, it is best suited for blood-based diseases.

Hematopoietic stem cells have been the main targets of *ex vivo* gene therapy to treat diseases such as β-thalassemia and sickle cell disease (SCD). Both result from DNA mutations in the beta-globin gene, which is required for the normal functioning of hemoglobin in red blood cells. β-thalassemia and SCD can actually both be cured by bone marrow transplantation, but the shortage of willing donors that can match the recipient immunologically remains a big challenge. Gene therapy is especially helpful in that patients serve as both recipient *and* donor of the stem cells, eliminating the need to find donors and the risk of a reverse immunological reaction. β-globin–expressing vectors can be added to the patient's own hematopoietic stem cells and allow for the production of corrected red blood cells for the life of the patient (Hoban et al., 2016).

Ex vivo therapy is also used in immunotherapy, where a patient's own immune cells are harvested and modified to fight diseases, such as cancer. Of note, Kymriah, an FDA-approved treatment developed by Novartis is used for acute lymphoblastic leukemia. In immunotherapy, the T cells of a patient are harvested and engineered to sprout special structures called chimeric antigen receptors (CAR) on their surface (Gonçalves & Paiva, 2017). When these CAR T cells are reinjected into the patient, the receptors help the T cells identify and attack cancer cells throughout the body.

***In vivo* gene therapy**

In vivo gene therapy has its advantages and disadvantages compared to *ex vivo*. It avoids the tedious procedures such as cell collection, culture, manipulation, and transplantation required for *ex-vivo* gene therapy (Dunbar et al., 2018). However, *in vivo* delivery requires tissue or cell type-specific targeting and local delivery. We have seen successful clinical trials that deliver genes to the liver, eye, and even the brain, laying foundations for other tissue targets.

The eye is both small and accessible as a delivery target. After years of experimentation, first in dogs, then children, Jean Bennett and her collaborator Katherine High, successfully delivered gene therapy using AAV2 to patients with inherited retinal dystrophy caused by RPE65 mutations (Russell et al., 2017). The therapy, Luxturna, received final approval from FDA in 2017 as the first *in vivo* gene therapy drug. In addition to eyes, *in vivo* gene therapy has also targeted CNS and neuromuscular disorders. A landmark study by Brian Kasper reported success with a newly engineered virus called AAV9 that had traversed the blood-brain barrier in neonatal mice (Foust et al., 2009). Kaspar's colleague Mendell launched a trial in 2014, using the intravenous administration of AAV9 for the treatment of spinal muscular atrophy type I (SMA1) in 15 patients. As reported in 2017, all patients showed rapid increases in motor function due to increased levels of the SMN protein (Mendell et al., 2017). FDA approved Zolgensma, the first gene therapy to treat SMA, in 2019.

5.2.4 CRISPR-based gene therapy

Traditional gene therapy, as in the cases discussed above, is limited to providing a functional copy of a gene, which means that it can mediate only one type of gene modification, that is "gene addition" (Dunbar et al., 2018). It does not fix the diseased gene itself. Genome editing, on the

other hand, can “repair” the diseased gene and conduct other gene modifications, such as gene deletion. Nevertheless, there are limits to what CRISPR can do. Some diseases are not caused by genetic mutations in a single gene, like schizophrenia or Alzheimer’s, where many genes are implicated and interacting in a way we are yet to understand.

Notably, all of the CRISPR-mediated gene therapies under development seek to treat patients through somatic cell modification, which affects only the individual who receives the treatment. Germline editing, on the other hand, would introduce genetic changes in eggs, sperms, or embryos. These changes would be passed on to future generations. The unique ethical and regulatory challenges associated with human germline editing are discussed in the later sections.

The first CRISPR Phase 1 clinical trial using CRISPR/Cas9 in the US was to edit autologous T cells for cancer immunotherapy to treat three patients with advanced cancer (Baylis & McLeod, 2018). The approach is closely related to CAR T cell therapy, which engineers autologous T cells to target antigens expressed on the surface of tumor cells. But it has some key differences. CIRSPR/Cas9 was used to remove three genes that encode the α and β chains of the endogenous T cell receptor (TCR) and the programmed cell death-1 (PD-1) protein, since removing them are believed to improve the function and persistence of engineered T cells (Stadtmauer et al., 2020). The T cells were edited *ex vivo* and then transduced with lentivirus to deliver a gene encoding a TCR specific for a NY-ESO-1 antigen, which serves as a therapeutic target. The engineered cells were administered to patients and were well tolerated. This shows that CAR T therapy combined with CRISPR/Cas9-mediated gene editing has the potential to improve the efficacy and safety of engineered T cells. This successful trial using CRISPR-mediated gene therapy encourages future CRISPR-engineered cancer immunotherapies.

CRISPR clinical trial was also conducted to treat patients with sickle-cell disease and later β-thalassemia. In July 2019, CRISPR Therapeutics together with Vertex Pharmaceuticals launched a CRISPR-based trial, using CRISPR/Cas9 to disrupt an enhancer for the BCL11A gene, which would increase fetal hemoglobin (HbF) levels and lead to therapeutic benefits for SCD. In the trial, *ex vivo* CRISPR-edited hematopoietic stem cells with disrupted BCL11A are delivered by IV injection. Initial results of the clinical trial are promising with 99.8% red blood cells expressing HbF after 9 months of the therapy (Ledford, 2020).

In addition to the *ex vivo* approaches summarized above, *in vivo* gene therapy also made strides. The first *in vivo* delivery of CRISPR/Cas9 was in patients with leber congenital amaurosis (LCA) type 10 in 2019. LCA type 10 is caused by a single letter mutation in a gene called CEP290 in both alleles. The loss of function mutation leads to degeneration of the outer segment of the photoreceptors, resulting in childhood blindness with no treatment options available. The therapy, called EDIT-101, uses AAV5 to deliver CRISPR/Cas9 directly into the retina to correct the mutation to restore normal gene splicing, thus producing the normal protein (Maeder et al., 2019). This was the first time CRISPR had been injected directly into a human patient, as opposed to the *ex vivo* approach employed by CRISPR Therapeutics. With the many successes in CRISPR-based therapeutics, it is without doubt that such therapies will continue to improve and benefit more patients in need.

5.3 Technical challenges and concerns

Although the potential of CRISPR-mediated gene therapy is enormous, there are many technical, economic, ethical and regulatory challenges that would need to be considered. The section below highlights some of the major technical challenges of the CRISPR/Cas system in therapeutic contexts.

5.3.1 Off-target effects

A major concern for implementing CRISPR-mediated gene therapy is the off-target effects. These occur when CRISPR-induced DNA cutting and repair happens at locations not intended for modification, usually sites that are close to the target editing site or a closely-related sequence (Doudna, 2020). Off-target effects are not unique for CRISPR. Virtually all medical drugs have some kind of side effects, but as long as the intended effects outweigh the risks, it is still worthwhile. However, what is unique about CRISPR is that any off-target DNA sequence, once edited, is irreversibly changed. Inaccurate editing could be harmless, but there is the chance that off-target edits could be devastating, switching on proto-oncogenes or potentially deactivating a tumor suppressor gene, leading to cancer. Although the likelihood that these events occur is small, it is critical to carefully detect and monitor such effects.

Scientists have devised many ways to address this concern though, including optimizing the guide RNA and designing Cas9 variants that have reduced off-target effects. The risks of introducing DNA cleavage have also spurred the development of other modification strategies that do not involve double-stranded DNA breaks. Catalytically dead Cas9 lacking endonuclease activity has been used to repurpose CRISPR to control gene expression in cells (Qi et al., 2013). CRISPR/Cas9 system has also been adapted to mediate epigenetic modifications (X. S. Liu et al., 2016). In

addition, base editing can catalyze any single-nucleotide changes without inducing a break in the DNA, limiting off-target effects (Komor et al., 2016).

5.3.2 Other concerns

Another safety issue is the immunogenicity of Cas proteins given that they are derived from bacteria. It is shown that many individuals carry anti-Cas9 antibodies, suggesting that they have been previously exposed to bacteria that have a CRISPR system (Charlesworth et al., 2019). These antibodies can target against CRISPR components to cause inflammation and perhaps more severe immune responses. Another report came out recently, suggesting cells that are genome edited with CRISPR can select against the function of p53, which is associated with rapid cell growth and cancer once disrupted (Haapaniemi et al., 2018). However, many different methods can minimize the risk of an unwanted immune response, such as selecting Cas9 enzymes from different bacteria or changing the surface of the protein to make it less immunogenic (Mehta & Merkel, 2020). Delivery has always been deemed as the biggest bottleneck to somatic-cell genome editing (Qi et al., 2013). Similar to traditional gene therapy, CRISPR-Cas machinery has to be delivered into the cells that are in need of genetic repair for them to be useful. Much remains to be investigated on how to make delivery more specific and effective.

Together, researchers have made great strides in developing new CRISPR technologies with improved specificity and safety. Armed with these tools, we are poised to apply them to cure a variety of genetic diseases including cancer. However, many technical challenges remain, including preventing off-target effects, immune responses, and oncogenesis. Thorough risk assessments, further preclinical studies in animal models, and meticulous designs of clinical trials would be necessary for these therapies to have a broad impact on human health. Gene therapy

based on genome editing is still in its infancy, but given the success so far, it will not take us long to overcome these technical challenges. Beyond technical challenges, economic, ethical, and regulatory questions remain and are discussed in the following section.

5.4 Economic, ethical, and regulatory implications of CRISPR in gene therapy

5.4.1 Economic implications

Gene therapy options have typically been developed to address rare genetic diseases with unmet medical needs. Unlike a traditional drug that can be sold repeatedly to thousands or millions of patients, gene therapy may only be applicable to a few patients and it is usually administered one-time or very infrequently compared to pills that are prescribed daily. Therefore, biotech companies usually charge a high price to recoup the substantial costs of development and testing. Most notably, Novartis priced their one-time therapy for spinal muscle atrophy (SMA), Zolgensma, at more than $2 million (Pearson et al., 2019). These treatments are seldom covered by insurance providers (R. C. Wilson & Carroll, 2019). Without insurance, the high price tag of such treatments essentially limits access to a select few patients who can afford them. Would this exacerbate the inequality in society where only people with the resources can enjoy the benefits of such treatments? Millions of people have SCD, especially in Africa, where access to bone marrow transplantation or traditional gene therapy is basically nonexistent, let alone CRISPR-based therapy. Affordability, especially in developing countries remains a huge hurdle to overcome before such therapies can make a broad impact.

5.4.2 Ethical controversies

Ethically speaking, gene therapy applied to somatic cells usually avoids the ethical issues inherent in germline editing, since the changes can’t be passed down to future generations. Germline editing,

however, introduces changes that are heritable. Although germline editing has been widely used in animals such as mice, rats, and monkeys and also in human embryos for research purposes, it remains highly controversial for therapeutic intent. Research has been done in both nonviable and viable human embryos (Fogarty et al., 2017; Liang et al., 2015), but none of the published work involves implantation of the edited embryos to initiate a pregnancy. However, one such clinical work carried out by He Jiankui in China was reported at the Second International Summit on Human Genome Ethics in November 2018, resulting in an international condemnation given its violations of ethical and scientific guidelines. It involved fertilized eggs from a couple that resulted in the birth of two twin girls. He attempted to disrupt a gene called CCR5, which encodes a receptor that HIV uses to enter cells, aiming to prevent the children from acquiring HIV. The work of He serves as an example for the relevant discussions below.

Addressing unmet medical needs

In the work of He, the stated goal was to confer HIV resistance. However, enhancing protection against HIV is not the same as correcting a disease-causing mutation. Given the fact that HIV prevention could be done via sperm washing to drive down the rate of HIV infection to near zero, preventing HIV is not an unmet medical need and cannot be justified. This example raises an important question: under what circumstances would clinical germline editing ever be considered? There are some situations where germline editing would be the only way to guarantee that children would be born free from a genetic disease. For example, both parents are homozygous for a severe disease-causing mutation such as cystic fibrosis, and the child through natural reproduction would have no way to avoid inheriting two mutated copies. In such cases, germline editing would seem justifiable from a medical-need perspective.

The question as to where to draw the line between an unmet medical need and an enhancement has become a trite debate, albeit an important one. It is medically defensible to use gene therapy for debilitating diseases, such as cystic fibrosis or sickle cell anemia, but what about gene edits that would target the prevention of diabetes, Alzheimer's, or reduced levels of cholesterol? Genes implicated in these diseases may also affect cognitive function and physical appearance, bordering on enhancement. Would this be considered as ethically acceptable? These are morally ambiguous and difficult questions and must be deliberated carefully, both for heritable and somatic edits.

Impact of new mutations introduced to the human population

It is challenging to modify disease risk by replacing genetic variants with alternative ones. First of all, there is the risk of failing to make the desired change. The work of He illustrates this point. The Δ32 mutation in the gene CCR5 occurs naturally, especially in northern European populations. HIV cannot infect the cells that are homozygous for the Δ32 alleles. However, the mutations introduced by He did not produce the Δ32 deletion, raising concerns about the effects of these man-made genetic alterations that have never been seen before in humans or tested in an animal model. Second, even if He had edited the CCR5 gene to replicate the naturally occurring Δ32 mutation, this gene variant may increase the risk of other diseases. The Δ32 mutation is present in northern Europeans, but there are almost no people in China with this mutation (Maeder et al., 2019). There is the possibility that the Δ32 mutation might have some other impact on health, hence it is rarely seen in Asia. This also concerns the case of sickle cell anemia in African populations, where heterozygotes confer a selective advantage of being protected from malaria. Together, this highlights another issue: How can we be sure that editing any gene will not have

some unforeseen effects? Given our current state of knowledge, understanding the effects of any genetic change will require extensive study. Even so, considerable uncertainty would still remain.

Informed consent

The issue of informed consent is one of the many criticisms people had when evaluating He's work. He personally obtained informed consent from the volunteer couples, which violates accepted ethical standards that an unbiased independent third party should conduct the informed consent process. In addition, CRISPR/Cas system is still in its early stages and even experts are still grappling with the risks and its long-term consequences. Without a reasonably complete understanding of the technology and the risks, can patients provide fully informed consent? What about unborn children? They will never have a chance to consent to a gene-edited version of themselves.

Broad societal consensus

He's work received wide criticism also because he did not conduct his work transparently with only a few people aware of his plan. Concerns of other scientists, clinicians, ethicists, and regulators need to be evaluated. The wider public's opinions also need to be heard. Given the technical nature of such technologies, it is easy to dismiss the general public's opinion as ill-informed on account of their lack of scientific understanding. However, a diversity of viewpoints is critical, both lay and expert, not just for the sake of it, but also because sometimes the risks imagined by experts are circumscribed (JB Hurlbut, 2015). Hence, broad engagement from a variety of stakeholders and voices are required and such engagement should be mediated with public education to allow for more equity in the process.

5.4.3 Current guidelines and regulations of gene editing

Gene editing is a clear example of scientific advancements outpacing regulation. On one hand, without clear regulations, a *laissez-faire* approach would lead to irresponsible incidents like He's again. By being so effective and easy to use, CRISPR-based gene editing may also be abused or employed for sinister purposes. On the other hand, strict regulations could stymie innovation. In addition, any prohibitions in one country would effectively cede leadership in this area to other nations with more lax regulations.

In 2017, the National Academies of Science, Engineering and Medicine (NASEM) released a detailed report on human genome editing (National Academies of Sciences, Engineering, and Medicine (U.S.), 2017). It concludes that clinical trials of genome editing in somatic cells to treat or prevent disease should continue, provided that the ethical norms and existing regulatory requirements are met. For germline editing that would result in inheritable changes by the next generation, the committee concludes that caution is needed, but that "caution does not mean prohibition". The NASEM report offered a top ten list of criteria to support any future use of clinical germline editing including no reasonable alternatives, restriction to preventing a serious disease, editing genes to known variants associated with ordinary health that are prevalent in the population, maximum transparency, and broader public input.

In light of He's affair, many leading scientists have called for a temporary global moratorium on all clinical uses of human germline editing to give scientists and other stakeholders time to examine the circumstances under which germline editing might be approved (Lander et al., 2019). More recently, the House of Representatives Appropriations Committee approved a rider that was once removed, barring the FDA from considering requests to approve any clinical trial "in which a

human embryo is intentionally created or modified to include a heritable genetic modification" (J. Kaiser, 2019). China has proposed to introduce a new regulation on gene editing in humans (Cyranoski, 2019). In the European Union, gene therapy is classified as advanced therapy medicinal products (ATMPs), regulated by the European Medicines Agency's Committee for Advanced Therapies. Recent updates include an action plan in place focusing on evaluation of ATMPs and soliciting multi-stakeholder feedback on the challenges (Detela & Lodge, 2019). In the UK, law inhibits using germline editing in assisted reproduction, although research is allowed under strict licensing conditions. Even with these efforts, it is unlikely that there will ever be a unanimous agreement on whether and how to use germline editing. Different people will approach the topic with different perspectives, histories, and cultural values. Nevertheless, governments should try their best to find the right balance between regulation and freedom.

On a global level, the National Academy of Science and the Royal Society have established an International Commission to define the circumstances under which germline genome editing should take place. The World Health Organization established an expert advisory committee tasked with "Developing global standards for governance and oversight of human genome editing." The committee had a kick-off meeting in March 2019 and proposed a global registry of germline editing research.

Moving forward

Although regulations in the biomedical field are usually governed by individual countries, germline gene editing is an exception in that the potential to alter the genetic code for all humanity transcends the geological and political boundaries. International coordination and agreements are especially important in addressing the profound challenges posed by the ethics of gene editing.

Regulations at the international level can help clarify and standardize the circumstances under which gene editing could be used. The efforts undertaken by the different organizations both at national and international levels are important first steps. With technologies as powerful and complex as CRISPR, the process of deliberation and risk assessment demands constant revisiting in light of new developments. In addition, the decisions ahead should not be just deferred to scientists or politicians. It is critical to widen the debate and include a variety of stakeholders, including scientists, physicians, ethicists, regulators, and the general public.

References

Anzalone, A. V., Randolph, P. B., Davis, J. R., Sousa, A. A., Koblan, L. W., Levy, J. M., Chen, P. J., Wilson, C., Newby, G. A., Raguram, A., & Liu, D. R. (2019). Search-and-replace genome editing without double-strand breaks or donor DNA. *Nature*, *576*(7785), 149–157. https://doi.org/10.1038/s41586-019-1711-4

Atchison, R. W., Casto, B. C., & Hammon, W. McD. (1965). Adenovirus-Associated Defective Virus Particles. *Science*, *149*(3685), 754–755. https://doi.org/10.1126/science.149.3685.754

Barrangou, R., Fremaux, C., Deveau, H., Richards, M., Boyaval, P., Moineau, S., Romero, D. A., & Horvath, P. (2007). CRISPR Provides Acquired Resistance Against Viruses in Prokaryotes. *Science*, *315*(5819), 1709–1712. https://doi.org/10.1126/science.1138140

Baylis, F., & McLeod, M. (2018). First-in-human Phase 1 CRISPR Gene Editing Cancer Trials: Are We Ready? *Current Gene Therapy*, *17*(4). https://doi.org/10.2174/1566523217666171121165935

Ben Mkaddem, S., Benhamou, M., & Monteiro, R. C. (2019). Understanding Fc Receptor Involvement in Inflammatory Diseases: From Mechanisms to New Therapeutic Tools. *Frontiers in Immunology*, *10*, 811. https://doi.org/10.3389/fimmu.2019.00811

Blaese, R. M., Culver, K. W., Miller, A. D., Carter, C. S., Fleisher, T., Clerici, M., Shearer, G., Chang, L., Chiang, Y., Tolstoshev, P., Greenblatt, J. J., Rosenberg, S. A., Klein, H., Berger, M., Mullen, C. A., Ramsey, W. J., Muul, L., Morgan, R. A., & Anderson, W. F. (1995). T Lymphocyte-Directed Gene Therapy for ADA- SCID: Initial Trial Results After 4 Years. *Science*, *270*(5235), 475–480. https://doi.org/10.1126/science.270.5235.475

Bordignon, C., Notarangelo, L. D., Nobili, N., Ferrari, G., Casorati, G., Panina, P., Mazzolari, E., Maggioni, D., Rossi, C., Servida, P., Ugazio, A. G., & Mavilio, F. (1995). Gene Therapy in Peripheral Blood Lymphocytes and Bone Marrow for ADA- Immunodeficient Patients. *Science*, *270*(5235), 470–475. https://doi.org/10.1126/science.270.5235.470

Brouns, S. J. J., Jore, M. M., Lundgren, M., Westra, E. R., Slijkhuis, R. J. H., Snijders, A. P. L., Dickman, M. J., Makarova, K. S., Koonin, E. V., & van der Oost, J. (2008). Small CRISPR RNAs Guide Antiviral Defense in Prokaryotes. *Science*, *321*(5891), 960–964. https://doi.org/10.1126/science.1159689

Buttgereit, A., Lelios, I., Yu, X., Vrohlings, M., Krakoski, N. R., Gautier, E. L., Nishinakamura, R., Becher, B., & Greter, M. (2016). Sall1 is a transcriptional regulator defining microglia identity and function. *Nature Immunology*, *17*(12), 1397–1406. https://doi.org/10.1038/ni.3585

Calver, A. R., Hall, A. C., Yu, W.-P., Walsh, F. S., Heath, J. K., Betsholtz, C., & Richardson, W. D. (1998). Oligodendrocyte Population Dynamics and the Role of PDGF In Vivo. *Neuron*, *20*(5), 869–882. https://doi.org/10.1016/S0896-6273(00)80469-9

Cantor, H., & Shinohara, M. L. (2009). Regulation of T-helper-cell lineage development by osteopontin: The inside story. *Nature Reviews Immunology*, *9*(2), 137–141. https://doi.org/10.1038/nri2460

Cavazzana-Calvo, M. (2000). Gene Therapy of Human Severe Combined Immunodeficiency (SCID)-X1 Disease. *Science*, *288*(5466), 669–672. https://doi.org/10.1126/science.288.5466.669

Chabas, D. (2001). The Influence of the Proinflammatory Cytokine, Osteopontin, on Autoimmune Demyelinating Disease. *Science*, *294*(5547), 1731–1735. https://doi.org/10.1126/science.1062960

Charlesworth, C. T., Deshpande, P. S., Dever, D. P., Camarena, J., Lemgart, V. T., Cromer, M. K., Vakulskas, C. A., Collingwood, M. A., Zhang, L., Bode, N. M., Behlke, M. A., Dejene, B., Cieniewicz, B., Romano, R., Lesch, B. J., Gomez-Ospina, N., Mantri, S., Pavel-Dinu, M., Weinberg, K. I., & Porteus, M. H. (2019). Identification of preexisting adaptive immunity to Cas9 proteins in humans. *Nature Medicine*, *25*(2), 249–254. https://doi.org/10.1038/s41591-018-0326-x

Chauhan, P., Hu, S., Sheng, W. S., Prasad, S., & Lokensgard, J. R. (2017). Modulation of Microglial Cell Fcγ Receptor Expression Following Viral Brain Infection. *Scientific Reports*, *7*(1), 41889. https://doi.org/10.1038/srep41889

Chiocchetti, A., Comi, C., Indelicato, M., Castelli, L., Mesturini, R., Bensi, T., Mazzarino, M. C., Giordano, M., D'Alfonso, S., Momigliano-Richiardi, P., Liguori, M., Zorzon, M., Amoroso, A., Trojano, M., Monaco, F., Leone, M., Magnani, C., & Dianzani, U. (2005). Osteopontin gene haplotypes correlate with multiple sclerosis development and progression. *Journal of Neuroimmunology*, *163*(1–2), 172–178. https://doi.org/10.1016/j.jneuroim.2005.02.020

Chung, J.-S., Sato, K., Dougherty, I. I., Cruz, P. D., & Ariizumi, K. (2007). DC-HIL is a negative regulator of T lymphocyte activation. *Blood*, *109*(10), 4320–4327. https://doi.org/10.1182/blood-2006-11-053769

Cong, L., Ran, F. A., Cox, D., Lin, S., Barretto, R., Habib, N., Hsu, P. D., Wu, X., Jiang, W., Marraffini, L. A., & Zhang, F. (2013). Multiplex Genome Engineering Using CRISPR/Cas Systems. *Science*, *339*(6121), 819–823. https://doi.org/10.1126/science.1231143

Cunningham, C. L., Martinez-Cerdeno, V., & Noctor, S. C. (2013). Microglia Regulate the Number of Neural Precursor Cells in the Developing Cerebral Cortex. *Journal of Neuroscience*, *33*(10), 4216–4233. https://doi.org/10.1523/JNEUROSCI.3441-12.2013

Cyranoski, D. (2019). China set to introduce gene-editing regulation following CRISPR-baby furore. *Nature*, d41586-019-01580–01581. https://doi.org/10.1038/d41586-019-01580-1

Detela, G., & Lodge, A. (2019). EU Regulatory Pathways for ATMPs: Standard, Accelerated and Adaptive Pathways to Marketing Authorisation. *Molecular Therapy - Methods & Clinical Development*, *13*, 205–232. https://doi.org/10.1016/j.omtm.2019.01.010

Doudna, J. A. (2020). The promise and challenge of therapeutic genome editing. *Nature*, *578*(7794), 229–236. https://doi.org/10.1038/s41586-020-1978-5

Dunbar, C. E., High, K. A., Joung, J. K., Kohn, D. B., Ozawa, K., & Sadelain, M. (2018). Gene therapy comes of age. *Science*, *359*(6372), eaan4672. https://doi.org/10.1126/science.aan4672

Duvernoy, H. M., & Risold, P.-Y. (2007). The circumventricular organs: An atlas of comparative anatomy and vascularization. *Brain Research Reviews*, *56*(1), 119–147. https://doi.org/10.1016/j.brainresrev.2007.06.002

Estes, M. L., & McAllister, A. K. (2015). Immune mediators in the brain and peripheral tissues in autism spectrum disorder. *Nature Reviews Neuroscience*, *16*(8), 469–486. https://doi.org/10.1038/nrn3978

Finer, M., & Glorioso, J. (2017). A brief account of viral vectors and their promise for gene therapy. *Gene Therapy*, *24*(1), 1–2. https://doi.org/10.1038/gt.2016.71

Fogarty, N. M. E., McCarthy, A., Snijders, K. E., Powell, B. E., Kubikova, N., Blakeley, P., Lea, R., Elder, K., Wamaitha, S. E., Kim, D., Maciulyte, V., Kleinjung, J., Kim, J.-S., Wells, D., Vallier, L., Bertero, A., Turner, J. M. A., & Niakan, K. K. (2017). Genome editing reveals a role for OCT4 in human embryogenesis. *Nature*, *550*(7674), 67–73. https://doi.org/10.1038/nature24033

Foust, K. D., Nurre, E., Montgomery, C. L., Hernandez, A., Chan, C. M., & Kaspar, B. K. (2009). Intravascular AAV9 preferentially targets neonatal neurons and adult astrocytes. *Nature Biotechnology*, *27*(1), 59–65. https://doi.org/10.1038/nbt.1515

Fridman, W. H. (1991). Fc receptors and immunoglobulin binding factors [1]. *The FASEB Journal*, *5*(12), 2684–2690. https://doi.org/10.1096/fasebj.5.12.1916092

Ginhoux, F., Greter, M., Leboeuf, M., Nandi, S., See, P., Gokhan, S., Mehler, M. F., Conway, S. J., Ng, L. G., Stanley, E. R., Samokhvalov, I. M., & Merad, M. (2010). Fate Mapping Analysis Reveals That Adult Microglia Derive from Primitive Macrophages. *Science*, *330*(6005), 841–845. https://doi.org/10.1126/science.1194637

Ginhoux, Florent, & Prinz, M. (2015). Origin of Microglia: Current Concepts and Past Controversies. *Cold Spring Harbor Perspectives in Biology*, *7*(8), a020537. https://doi.org/10.1101/cshperspect.a020537

Goldmann, T., Wieghofer, P., Jordão, M. J. C., Prutek, F., Hagemeyer, N., Frenzel, K., Amann, L., Staszewski, O., Kierdorf, K., Krueger, M., Locatelli, G., Hochgerner, H., Zeiser, R., Epelman, S., Geissmann, F., Priller, J., Rossi, F. M. V., Bechmann, I., Kerschensteiner, M., … Prinz, M. (2016). Origin, fate and dynamics of macrophages at central nervous system interfaces. *Nature Immunology*, *17*(7), 797–805. https://doi.org/10.1038/ni.3423

Gomez Perdiguero, E., Klapproth, K., Schulz, C., Busch, K., Azzoni, E., Crozet, L., Garner, H., Trouillet, C., de Bruijn, M. F., Geissmann, F., & Rodewald, H.-R. (2015). Tissue-resident macrophages originate from yolk-sac-derived erythro-myeloid progenitors. *Nature*, *518*(7540), 547–551. https://doi.org/10.1038/nature13989

Gonçalves, G. A. R., & Paiva, R. de M. A. (2017). Gene therapy: Advances, challenges and perspectives. *Einstein (São Paulo)*, *15*(3), 369–375. https://doi.org/10.1590/s1679-45082017rb4024

Graciarena, M., Seiffe, A., Nait-Oumesmar, B., & Depino, A. M. (2019). Hypomyelination and Oligodendroglial Alterations in a Mouse Model of Autism Spectrum Disorder. *Frontiers in Cellular Neuroscience*, *12*, 517. https://doi.org/10.3389/fncel.2018.00517

Guitart, J. R., Johnson, J. L., & Chien, W. W. (2016). Research Techniques Made Simple: The Application of CRISPR-Cas9 and Genome Editing in Investigative Dermatology. *Journal of Investigative Dermatology*, *136*(9), e87–e93. https://doi.org/10.1016/j.jid.2016.06.007

Haapaniemi, E., Botla, S., Persson, J., Schmierer, B., & Taipale, J. (2018). CRISPR–Cas9 genome editing induces a p53-mediated DNA damage response. *Nature Medicine*, *24*(7), 927–930. https://doi.org/10.1038/s41591-018-0049-z

Hagemeyer, N., Hanft, K.-M., Akriditou, M.-A., Unger, N., Park, E. S., Stanley, E. R., Staszewski, O., Dimou, L., & Prinz, M. (2017). Microglia contribute to normal myelinogenesis and to oligodendrocyte progenitor maintenance during adulthood. *Acta Neuropathologica*, *134*(3), 441–458. https://doi.org/10.1007/s00401-017-1747-1

Haimon, Z., Volaski, A., Orthgiess, J., Boura-Halfon, S., Varol, D., Shemer, A., Yona, S., Zuckerman, B., David, E., Chappell-Maor, L., Bechmann, I., Gericke, M., Ulitsky, I., & Jung, S. (2018). Re-evaluating microglia expression profiles using RiboTag and cell

isolation strategies. *Nature Immunology*, *19*(6), 636–644. https://doi.org/10.1038/s41590-018-0110-6

Hammond, T. R., Dufort, C., Dissing-Olesen, L., Giera, S., Young, A., Wysoker, A., Walker, A. J., Gergits, F., Segel, M., Nemesh, J., Marsh, S. E., Saunders, A., Macosko, E., Ginhoux, F., Chen, J., Franklin, R. J. M., Piao, X., McCarroll, S. A., & Stevens, B. (2019). Single-Cell RNA Sequencing of Microglia throughout the Mouse Lifespan and in the Injured Brain Reveals Complex Cell-State Changes. *Immunity*, *50*(1), 253-271.e6. https://doi.org/10.1016/j.immuni.2018.11.004

Hastie, E., & Samulski, R. J. (2015). Adeno-Associated Virus at 50: A Golden Anniversary of Discovery, Research, and Gene Therapy Success—A Personal Perspective. *Human Gene Therapy*, *26*(5), 257–265. https://doi.org/10.1089/hum.2015.025

Hoban, M. D., Orkin, S. H., & Bauer, D. E. (2016). Genetic treatment of a molecular disorder: Gene therapy approaches to sickle cell disease. *Blood*, *127*(7), 839–848. https://doi.org/10.1182/blood-2015-09-618587

Huang, J., Ma, W., & Yokoyama, S. (2012). Expression and immunolocalization of Gpnmb, a glioma-associated glycoprotein, in normal and inflamed central nervous systems of adult rats. *Brain and Behavior*, *2*(2), 85–96. https://doi.org/10.1002/brb3.39

Hughes, A. N., & Appel, B. (2020). Microglia phagocytose myelin sheaths to modify developmental myelination. *Nature Neuroscience*, *23*(9), 1055–1066. https://doi.org/10.1038/s41593-020-0654-2

Humbert, O., Davis, L., & Maizels, N. (2012). Targeted gene therapies: Tools, applications, optimization. *Critical Reviews in Biochemistry and Molecular Biology*, *47*(3), 264–281. https://doi.org/10.3109/10409238.2012.658112

Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J. A., & Charpentier, E. (2012). A Programmable Dual-RNA-Guided DNA Endonuclease in Adaptive Bacterial Immunity. *Science*, *337*(6096), 816–821. https://doi.org/10.1126/science.1225829

Jordão, M. J. C., Sankowski, R., Brendecke, S. M., Sagar, Locatelli, G., Tai, Y.-H., Tay, T. L., Schramm, E., Armbruster, S., Hagemeyer, N., Groß, O., Mai, D., Çiçek, Ö., Falk, T., Kerschensteiner, M., Grün, D., & Prinz, M. (2019). Single-cell profiling identifies myeloid cell subsets with distinct fates during neuroinflammation. *Science*, *363*(6425), eaat7554. https://doi.org/10.1126/science.aat7554

Kaiser, J. (2019). Update: House spending panel restores U.S. ban on gene-edited babies. *Science*. https://doi.org/10.1126/science.aay1607

Kaiser, T., & Feng, G. (2019). Tmem119-EGFP and Tmem119-CreERT2 Transgenic Mice for Labeling and Manipulating Microglia. *Eneuro*, *6*(4), ENEURO.0448-18.2019. https://doi.org/10.1523/ENEURO.0448-18.2019

Keren-Shaul, H., Spinrad, A., Weiner, A., Matcovitch-Natan, O., Dvir-Szternfeld, R., Ulland, T. K., David, E., Baruch, K., Lara-Astaiso, D., Toth, B., Itzkovitz, S., Colonna, M., Schwartz, M., & Amit, I. (2017). A Unique Microglia Type Associated with Restricting Development of Alzheimer's Disease. *Cell*, *169*(7), 1276-1290.e17. https://doi.org/10.1016/j.cell.2017.05.018

Kessaris, N., Fogarty, M., Iannarelli, P., Grist, M., Wegner, M., & Richardson, W. D. (2006). Competing waves of oligodendrocytes in the forebrain and postnatal elimination of an embryonic lineage. *Nature Neuroscience*, *9*(2), 173–179. https://doi.org/10.1038/nn1620

Kim, J.-S., Kolesnikov, M., Peled-Hajaj, S., Scheyltjens, I., Xia, Y., Trzebanski, S., Haimon, Z., Shemer, A., Lubart, A., Van Hove, H., Chappell-Maor, L., Boura-Halfon, S., Movahedi,

K., Blinder, P., & Jung, S. (2021). A Binary Cre Transgenic Approach Dissects Microglia and CNS Border-Associated Macrophages. *Immunity*, *54*(1), 176-190.e7. https://doi.org/10.1016/j.immuni.2020.11.007

Komor, A. C., Kim, Y. B., Packer, M. S., Zuris, J. A., & Liu, D. R. (2016). Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage. *Nature*, *533*(7603), 420–424. https://doi.org/10.1038/nature17946

Lander, E. S., Baylis, F., Zhang, F., Charpentier, E., Berg, P., Bourgain, C., Friedrich, B., Joung, J. K., Li, J., Liu, D., Naldini, L., Nie, J.-B., Qiu, R., Schoene-Seifert, B., Shao, F., Terry, S., Wei, W., & Winnacker, E.-L. (2019). Adopt a moratorium on heritable genome editing. *Nature*, *567*(7747), 165–168. https://doi.org/10.1038/d41586-019-00726-5

Ledford, H. (2020). Quest to use CRISPR against disease gains ground. *Nature*, *577*(7789), 156–156. https://doi.org/10.1038/d41586-019-03919-0

Li, H., Yang, Y., Hong, W., Huang, M., Wu, M., & Zhao, X. (2020). Applications of genome editing technology in the targeted therapy of human diseases: Mechanisms, advances and prospects. *Signal Transduction and Targeted Therapy*, *5*(1), 1. https://doi.org/10.1038/s41392-019-0089-y

Li, Q., Cheng, Z., Zhou, L., Darmanis, S., Neff, N. F., Okamoto, J., Gulati, G., Bennett, M. L., Sun, L. O., Clarke, L. E., Marschallinger, J., Yu, G., Quake, S. R., Wyss-Coray, T., & Barres, B. A. (2019). Developmental Heterogeneity of Microglia and Brain Myeloid Cells Revealed by Deep Single-Cell RNA Sequencing. *Neuron*, *101*(2), 207-223.e10. https://doi.org/10.1016/j.neuron.2018.12.006

Liang, P., Xu, Y., Zhang, X., Ding, C., Huang, R., Zhang, Z., Lv, J., Xie, X., Chen, Y., Li, Y., Sun, Y., Bai, Y., Songyang, Z., Ma, W., Zhou, C., & Huang, J. (2015). CRISPR/Cas9-mediated gene editing in human tripronuclear zygotes. *Protein & Cell*, *6*(5), 363–372. https://doi.org/10.1007/s13238-015-0153-5

Liu, X. S., Wu, H., Ji, X., Stelzer, Y., Wu, X., Czauderna, S., Shu, J., Dadon, D., Young, R. A., & Jaenisch, R. (2016). Editing DNA Methylation in the Mammalian Genome. *Cell*, *167*(1), 233-247.e17. https://doi.org/10.1016/j.cell.2016.08.056

Liu, Y., Given, K. S., Dickson, E. L., Owens, G. P., Macklin, W. B., & Bennett, J. L. (2019). Concentration-dependent effects of CSF1R inhibitors on oligodendrocyte progenitor cells ex vivo and in vivo. *Experimental Neurology*, *318*, 32–41. https://doi.org/10.1016/j.expneurol.2019.04.011

Madisen, L., Zwingman, T. A., Sunkin, S. M., Oh, S. W., Zariwala, H. A., Gu, H., Ng, L. L., Palmiter, R. D., Hawrylycz, M. J., Jones, A. R., Lein, E. S., & Zeng, H. (2010). A robust and high-throughput Cre reporting and characterization system for the whole mouse brain. *Nature Neuroscience*, *13*(1), 133–140. https://doi.org/10.1038/nn.2467

Maeder, M. L., & Gersbach, C. A. (2016). Genome-editing Technologies for Gene and Cell Therapy. *Molecular Therapy*, *24*(3), 430–446. https://doi.org/10.1038/mt.2016.10

Maeder, M. L., Stefanidakis, M., Wilson, C. J., Baral, R., Barrera, L. A., Bounoutas, G. S., Bumcrot, D., Chao, H., Ciulla, D. M., DaSilva, J. A., Dass, A., Dhanapal, V., Fennell, T. J., Friedland, A. E., Giannoukos, G., Gloskowski, S. W., Glucksmann, A., Gotta, G. M., Jayaram, H., … Jiang, H. (2019). Development of a gene-editing approach to restore vision loss in Leber congenital amaurosis type 10. *Nature Medicine*, *25*(2), 229–233. https://doi.org/10.1038/s41591-018-0327-9

Marín-Teva, J. L., Dusart, I., Colin, C., Gervais, A., van Rooijen, N., & Mallat, M. (2004). Microglia Promote the Death of Developing Purkinje Cells. *Neuron*, *41*(4), 535–547. https://doi.org/10.1016/S0896-6273(04)00069-8

Marques, S., Zeisel, A., Codeluppi, S., van Bruggen, D., Mendanha Falcao, A., Xiao, L., Li, H., Haring, M., Hochgerner, H., Romanov, R. A., Gyllborg, D., Munoz-Manchado, A. B., La Manno, G., Lonnerberg, P., Floriddia, E. M., Rezayee, F., Ernfors, P., Arenas, E., Hjerling-Leffler, J., … Castelo-Branco, G. (2016). Oligodendrocyte heterogeneity in the mouse juvenile and adult central nervous system. *Science*, *352*(6291), 1326–1329. https://doi.org/10.1126/science.aaf6463

Masuda, T., Amann, L., Sankowski, R., Staszewski, O., Lenz, M., d´Errico, P., Snaidero, N., Costa Jordão, M. J., Böttcher, C., Kierdorf, K., Jung, S., Priller, J., Misgeld, T., Vlachos, A., Meyer-Luehmann, M., Knobeloch, K.-P., & Prinz, M. (2020). Novel Hexb-based tools for studying microglia in the CNS. *Nature Immunology*, *21*(7), 802–815. https://doi.org/10.1038/s41590-020-0707-4

McKinsey, G. L., Lizama, C. O., Keown-Lang, A. E., Niu, A., Santander, N., Larpthaveesarp, A., Chee, E., Gonzalez, F. F., & Arnold, T. D. (2020). A new genetic strategy for targeting microglia in development and disease. *ELife*, *9*, e54590. https://doi.org/10.7554/eLife.54590

Mehta, A., & Merkel, O. M. (2020). Immunogenicity of Cas9 Protein. *Journal of Pharmaceutical Sciences*, *109*(1), 62–67. https://doi.org/10.1016/j.xphs.2019.10.003

Mendell, J. R., Al-Zaidy, S., Shell, R., Arnold, W. D., Rodino-Klapac, L. R., Prior, T. W., Lowes, L., Alfano, L., Berry, K., Church, K., Kissel, J. T., Nagendran, S., L'Italien, J., Sproule, D. M., Wells, C., Cardenas, J. A., Heitzer, M. D., Kaspar, A., Corcoran, S., … Kaspar, B. K. (2017). Single-Dose Gene-Replacement Therapy for Spinal Muscular Atrophy. *New England Journal of Medicine*, *377*(18), 1713–1722. https://doi.org/10.1056/NEJMoa1706198

Milone, M. C., & O'Doherty, U. (2018). Clinical use of lentiviral vectors. *Leukemia*, *32*(7), 1529–1541. https://doi.org/10.1038/s41375-018-0106-0

Mojica, F. J. M., D�ez-Villase�or, C., Garc�a-Mart�nez, J., & Soria, E. (2005). Intervening Sequences of Regularly Spaced Prokaryotic Repeats Derive from Foreign Genetic Elements. *Journal of Molecular Evolution*, *60*(2), 174–182. https://doi.org/10.1007/s00239-004-0046-3

Mrdjen, D., Pavlovic, A., Hartmann, F. J., Schreiner, B., Utz, S. G., Leung, B. P., Lelios, I., Heppner, F. L., Kipnis, J., Merkler, D., Greter, M., & Becher, B. (2018). High-Dimensional Single-Cell Mapping of Central Nervous System Immune Cells Reveals Distinct Myeloid Subsets in Health, Aging, and Disease. *Immunity*, *48*(2), 380-395.e6. https://doi.org/10.1016/j.immuni.2018.01.011

Nakahara, J., Tan-Takeuchi, K., Seiwa, C., Gotoh, M., Kaifu, T., Ujike, A., Inui, M., Yagi, T., Ogawa, M., Aiso, S., Takai, T., & Asou, H. (2003). Signaling via Immunoglobulin Fc Receptors Induces Oligodendrocyte Precursor Cell Differentiation. *Developmental Cell*, *4*(6), 841–852. https://doi.org/10.1016/S1534-5807(03)00155-2

Nasrabady, S. E., Rizvi, B., Goldman, J. E., & Brickman, A. M. (2018). White matter changes in Alzheimer's disease: A focus on myelin and oligodendrocytes. *Acta Neuropathologica Communications*, *6*(1), 22. https://doi.org/10.1186/s40478-018-0515-3

National Academies of Sciences, Engineering, and Medicine (U.S.) (Ed.). (2017). *Human genome editing: Science, ethics, and governance*. The National Academies Press.

Nemes-Baran, A. D., White, D. R., & DeSilva, T. M. (2020). Fractalkine-Dependent Microglial Pruning of Viable Oligodendrocyte Progenitor Cells Regulates Myelination. *Cell Reports*, *32*(7), 108047. https://doi.org/10.1016/j.celrep.2020.108047

Nimmerjahn, F., & Ravetch, J. V. (2008). Fcγ receptors as regulators of immune responses. *Nature Reviews Immunology*, *8*(1), 34–47. https://doi.org/10.1038/nri2206

Pearson, S. D., Thokala, P., Stevenson, M., & Rind, D. (2019). The Effectiveness and Value of Treatments for Spinal Muscular Atrophy: A Summary from the Institute for Clinical and Economic Review's New England Comparative Effectiveness Public Advisory Council. *Journal of Managed Care & Specialty Pharmacy*, *25*(12), 1300–1306. https://doi.org/10.18553/jmcp.2019.25.12.1300

Pickar-Oliver, A., & Gersbach, C. A. (2019). The next generation of CRISPR–Cas technologies and applications. *Nature Reviews Molecular Cell Biology*, *20*(8), 490–507. https://doi.org/10.1038/s41580-019-0131-5

Qi, L. S., Larson, M. H., Gilbert, L. A., Doudna, J. A., Weissman, J. S., Arkin, A. P., & Lim, W. A. (2013). Repurposing CRISPR as an RNA-Guided Platform for Sequence-Specific Control of Gene Expression. *Cell*, *152*(5), 1173–1183. https://doi.org/10.1016/j.cell.2013.02.022

Rabenstein, M., Vay, S. U., Flitsch, L. J., Fink, G. R., Schroeter, M., & Rueger, M. A. (2016). Osteopontin directly modulates cytokine expression of primary microglia and increases their survival. *Journal of Neuroimmunology*, *299*, 130–138. https://doi.org/10.1016/j.jneuroim.2016.09.009

Raper, S. E., Chirmule, N., Lee, F. S., Wivel, N. A., Bagg, A., Gao, G., Wilson, J. M., & Batshaw, M. L. (2003). Fatal systemic inflammatory response syndrome in a ornithine transcarbamylase deficient patient following adenoviral gene transfer. *Molecular Genetics and Metabolism*, *80*(1–2), 148–158. https://doi.org/10.1016/j.ymgme.2003.08.016

Rinaldi, M., Thomas, L., Mathieu, P., Carabias, P., Troncoso, M. F., Pasquini, J. M., Rabinovich, G. A., & Pasquini, L. A. (2016). Galectin-1 circumvents lysolecithin-induced demyelination through the modulation of microglial polarization/phagocytosis and oligodendroglial differentiation. *Neurobiology of Disease*, *96*, 127–143. https://doi.org/10.1016/j.nbd.2016.09.003

Ripoll, V. M., Irvine, K. M., Ravasi, T., Sweet, M. J., & Hume, D. A. (2007). *Gpnmb* Is Induced in Macrophages by IFN-γ and Lipopolysaccharide and Acts as a Feedback Regulator of Proinflammatory Responses. *The Journal of Immunology*, *178*(10), 6557–6566. https://doi.org/10.4049/jimmunol.178.10.6557

Roopenian, D. C., & Akilesh, S. (2007). FcRn: The neonatal Fc receptor comes of age. *Nature Reviews Immunology*, *7*(9), 715–725. https://doi.org/10.1038/nri2155

Russell, S., Bennett, J., Wellman, J. A., Chung, D. C., Yu, Z.-F., Tillman, A., Wittes, J., Pappas, J., Elci, O., McCague, S., Cross, D., Marshall, K. A., Walshire, J., Kehoe, T. L., Reichert, H., Davis, M., Raffini, L., George, L. A., Hudson, F. P., … Maguire, A. M. (2017). Efficacy and safety of voretigene neparvovec (AAV2-hRPE65v2) in patients with RPE65 -mediated inherited retinal dystrophy: A randomised, controlled, open-label, phase 3 trial. *The Lancet*, *390*(10097), 849–860. https://doi.org/10.1016/S0140-6736(17)31868-8

Saunders, A., Macosko, E. Z., Wysoker, A., Goldman, M., Krienen, F. M., de Rivera, H., Bien, E., Baum, M., Bortolin, L., Wang, S., Goeva, A., Nemesh, J., Kamitaki, N., Brumbaugh, S., Kulp, D., & McCarroll, S. A. (2018). Molecular Diversity and Specializations among

the Cells of the Adult Mouse Brain. *Cell*, *174*(4), 1015-1030.e16. https://doi.org/10.1016/j.cell.2018.07.028

Schafer, D. P., Lehrman, E. K., Kautzman, A. G., Koyama, R., Mardinly, A. R., Yamasaki, R., Ransohoff, R. M., Greenberg, M. E., Barres, B. A., & Stevens, B. (2012). Microglia Sculpt Postnatal Neural Circuits in an Activity and Complement-Dependent Manner. *Neuron*, *74*(4), 691–705. https://doi.org/10.1016/j.neuron.2012.03.026

Schafer, D. P., & Stevens, B. (2015). Microglia Function in Central Nervous System Development and Plasticity. *Cold Spring Harbor Perspectives in Biology*, *7*(10), a020545. https://doi.org/10.1101/cshperspect.a020545

Schlachetzki, F., Zhu, C., & Pardridge, W. M. (2002). Expression of the neonatal Fc receptor (FcRn) at the blood-brain barrier: Expression of FcRn at the blood-brain barrier. *Journal of Neurochemistry*, *81*(1), 203–206. https://doi.org/10.1046/j.1471-4159.2002.00840.x

Shechter, R., London, A., Varol, C., Raposo, C., Cusimano, M., Yovel, G., Rolls, A., Mack, M., Pluchino, S., Martino, G., Jung, S., & Schwartz, M. (2009). Infiltrating Blood-Derived Macrophages Are Vital Cells Playing an Anti-inflammatory Role in Recovery from Spinal Cord Injury in Mice. *PLoS Medicine*, *6*(7), e1000113. https://doi.org/10.1371/journal.pmed.1000113

Sierra, A., Abiega, O., Shahraz, A., & Neumann, H. (2013). Janus-faced microglia: Beneficial and detrimental consequences of microglial phagocytosis. *Frontiers in Cellular Neuroscience*, *7*. https://doi.org/10.3389/fncel.2013.00006

Stadtmauer, E. A., Fraietta, J. A., Davis, M. M., Cohen, A. D., Weber, K. L., Lancaster, E., Mangan, P. A., Kulikovskaya, I., Gupta, M., Chen, F., Tian, L., Gonzalez, V. E., Xu, J., Jung, I., Melenhorst, J. J., Plesa, G., Shea, J., Matlawski, T., Cervini, A., … June, C. H. (2020). CRISPR-engineered T cells in patients with refractory cancer. *Science*, *367*(6481), eaba7365. https://doi.org/10.1126/science.aba7365

Starossom, S. C., Mascanfroni, I. D., Imitola, J., Cao, L., Raddassi, K., Hernandez, S. F., Bassil, R., Croci, D. O., Cerliani, J. P., Delacour, D., Wang, Y., Elyaman, W., Khoury, S. J., & Rabinovich, G. A. (2012). Galectin-1 Deactivates Classically Activated Microglia and Protects from Inflammation-Induced Neurodegeneration. *Immunity*, *37*(2), 249–263. https://doi.org/10.1016/j.immuni.2012.05.023

Takai, T., Li, M., Sylvestre, D., Clynes, R., & Ravetch, J. V. (1994). FcR γ chain deletion results in pleiotrophic effector cell defects. *Cell*, *76*(3), 519–529. https://doi.org/10.1016/0092-8674(94)90115-5

Uddin, F., Rudin, C. M., & Sen, T. (2020). CRISPR Gene Therapy: Applications, Limitations, and Implications for the Future. *Frontiers in Oncology*, *10*, 1387. https://doi.org/10.3389/fonc.2020.01387

Uranova, N. A., Vikhreva, O. V., Rachmanova, V. I., & Orlovskaya, D. D. (2011). Ultrastructural Alterations of Myelinated Fibers and Oligodendrocytes in the Prefrontal Cortex in Schizophrenia: A Postmortem Morphometric Study. *Schizophrenia Research and Treatment*, *2011*, 1–13. https://doi.org/10.1155/2011/325789

Van Hove, H., Martens, L., Scheyltjens, I., De Vlaminck, K., Pombo Antunes, A. R., De Prijck, S., Vandamme, N., De Schepper, S., Van Isterdael, G., Scott, C. L., Aerts, J., Berx, G., Boeckxstaens, G. E., Vandenbroucke, R. E., Vereecke, L., Moechars, D., Guilliams, M., Van Ginderachter, J. A., Saeys, Y., & Movahedi, K. (2019). A single-cell atlas of mouse brain macrophages reveals unique transcriptional identities shaped by ontogeny and

tissue environment. *Nature Neuroscience*, *22*(6), 1021–1035. https://doi.org/10.1038/s41593-019-0393-4

Vanlandewijck, M., He, L., Mäe, M. A., Andrae, J., Ando, K., Del Gaudio, F., Nahar, K., Lebouvier, T., Laviña, B., Gouveia, L., Sun, Y., Raschperger, E., Räsänen, M., Zarb, Y., Mochizuki, N., Keller, A., Lendahl, U., & Betsholtz, C. (2018). A molecular atlas of cell types and zonation in the brain vasculature. *Nature*, *554*(7693), 475–480. https://doi.org/10.1038/nature25739

Vidarsson, G., Dekkers, G., & Rispens, T. (2014). IgG Subclasses and Allotypes: From Structure to Effector Functions. *Frontiers in Immunology*, *5*. https://doi.org/10.3389/fimmu.2014.00520

Villaseñor, R., Ozmen, L., Messaddeq, N., Grüninger, F., Loetscher, H., Keller, A., Betsholtz, C., Freskgård, P.-O., & Collin, L. (2016). Trafficking of Endogenous Immunoglobulins by Endothelial Cells at the Blood-Brain Barrier. *Scientific Reports*, *6*(1), 25658. https://doi.org/10.1038/srep25658

Wakselman, S., Bechade, C., Roumier, A., Bernard, D., Triller, A., & Bessis, A. (2008). Developmental Neuronal Death in Hippocampus Requires the Microglial CD11b Integrin and DAP12 Immunoreceptor. *Journal of Neuroscience*, *28*(32), 8138–8143. https://doi.org/10.1523/JNEUROSCI.1006-08.2008

Wang, D., Tai, P. W. L., & Gao, G. (2019). Adeno-associated virus vector as a platform for gene therapy delivery. *Nature Reviews Drug Discovery*, *18*(5), 358–378. https://doi.org/10.1038/s41573-019-0012-9

Wang, K. X., & Denhardt, D. T. (2008). Osteopontin: Role in immune regulation and stress responses. *Cytokine & Growth Factor Reviews*, *19*(5–6), 333–345. https://doi.org/10.1016/j.cytogfr.2008.08.001

Waxman, S. G. (1980). Determinants of conduction velocity in myelinated nerve fibers. *Muscle & Nerve*, *3*(2), 141–150. https://doi.org/10.1002/mus.880030207

Weterman, M. A. J., Ajubi, N., van Dinter, I. M. R., Degen, W. G. J., van Muijen, G. N. P., Ruiter, D. J., & Bloemers, H. P. J. (1995). Nmb, a novel gene, is expressed in low-metastatic human melanoma cell lines and xenografts. *International Journal of Cancer*, *60*(1), 73–81. https://doi.org/10.1002/ijc.2910600111

Wilson, M. J., Lindquist, J. A., & Trowsdale, J. (2000). DAP12 and KAP10 (DAP10)-Novel Transmembrane Adapter Proteins of the CD3ζ Family. *Immunologic Research*, *22*(1), 21–42. https://doi.org/10.1385/IR:22:1:21

Wilson, R. C., & Carroll, D. (2019). The Daunting Economics of Therapeutic Genome Editing. *The CRISPR Journal*, *2*(5), 280–284. https://doi.org/10.1089/crispr.2019.0052

Wirths, O., Breyhan, H., Marcello, A., Cotel, M.-C., Brück, W., & Bayer, T. A. (2010). Inflammatory changes are tightly associated with neurodegeneration in the brain and spinal cord of the APP/PS1KI mouse model of Alzheimer's disease. *Neurobiology of Aging*, *31*(5), 747–757. https://doi.org/10.1016/j.neurobiolaging.2008.06.011

Wlodarczyk, A., Holtman, I. R., Krueger, M., Yogev, N., Bruttger, J., Khorooshi, R., Benmamar-Badel, A., Boer-Bergsma, J. J., Martin, N. A., Karram, K., Kramer, I., Boddeke, E. W., Waisman, A., Eggen, B. J., & Owens, T. (2017). A novel microglial subset plays a key role in myelinogenesis in developing brain. *The EMBO Journal*, *36*(22), 3292–3308. https://doi.org/10.15252/embj.201696056

Wu, Z., Yang, H., & Colosi, P. (2010). Effect of Genome Size on AAV Vector Packaging. *Molecular Therapy*, *18*(1), 80–86. https://doi.org/10.1038/mt.2009.255

Yu, H., Liu, X., & Zhong, Y. (2017). The Effect of Osteopontin on Microglia. *BioMed Research International*, *2017*, 1–6. https://doi.org/10.1155/2017/1879437

Zhang, Y., Chen, K., Sloan, S. A., Bennett, M. L., Scholze, A. R., O'Keeffe, S., Phatnani, H. P., Guarnieri, P., Caneda, C., Ruderisch, N., Deng, S., Liddelow, S. A., Zhang, C., Daneman, R., Maniatis, T., Barres, B. A., & Wu, J. Q. (2014). An RNA-Sequencing Transcriptome and Splicing Database of Glia, Neurons, and Vascular Cells of the Cerebral Cortex. *Journal of Neuroscience*, *34*(36), 11929–11947. https://doi.org/10.1523/JNEUROSCI.1860-14.2014

Zhang, Yun, & Pardridge, W. M. (2001). Mediated efflux of IgG molecules from brain to blood across the blood–brain barrier. *Journal of Neuroimmunology*, *114*(1–2), 168–172. https://doi.org/10.1016/S0165-5728(01)00242-9

Zhao, X.-F., Alam, M. M., Liao, Y., Huang, T., Mathur, R., Zhu, X., & Huang, Y. (2019). Targeting Microglia Using Cx3cr1-Cre Lines: Revisiting the Specificity. *Eneuro*, *6*(4), ENEURO.0114-19.2019. https://doi.org/10.1523/ENEURO.0114-19.2019

Zuchero, J. B., & Barres, B. A. (2013). Intrinsic and extrinsic control of oligodendrocyte development. *Current Opinion in Neurobiology*, *23*(6), 914–920. https://doi.org/10.1016/j.conb.2013.06.005