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The G-protein coupled receptor GPR34 promotes the homeostatic state of microglia and restrains the disease-associated microglial response in an AD model

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eLife Assessment

This **valuable** study provides key insights into the role of the G protein-coupled receptor GPR34 in an Alzheimer's disease (AD) model. Notably, its findings differ, at least in part, from those of previous studies, although the underlying reasons for these discrepancies are not investigated or discussed. The data show that GPR34 deficiency enhances the transcriptional disease-associated microglia signature in microglia and provide **solid** evidence that myelin is a GPR34 ligand, although the relevance of myelin engulfment in AD is unclear. This study would be of interest for neuro-immunologists and AD clinicians.

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Abstract

Expression of the G protein coupled receptor GPR34 is highly enriched in microglia and has been reported to be downregulated in several brain disease contexts, including Alzheimers disease (AD) and multiple sclerosis (MS). GPR34 function is poorly understood, as is its role in regulation of microglial states. Using RNA-sequencing, we find that microglia from *Gpr34* knockout (KO) mouse brains exhibited a transcriptomic shift toward disease-associated microglia (DAM) and inflammatory profiles, partially resembling the microglial phenotype seen in 5xFAD AD model mice. Moreover, when *Gpr34* KO mice were crossed with 5xFAD mice, the DAM transcriptional profile of microglia and glial pathology were further enhanced beyond the already robust DAM signature driven by 5xFAD alone. This occurred without affecting amyloid plaque burden. Human stem cell-derived microglia (iMGLs) lacking *GPR34* showed reduced calcium (Ca²⁺) and phosphorylated ERK (pERK) signaling in response to stimulation with known GPR34 agonists (lyso-

phosphatidylserine (lysoPS) and myelin), as well as transcriptomic changes in immune regulation and cell proliferation related pathways. Interestingly, *GPR34* KO iMGLs were selectively impaired in phagocytosis of myelin but not amyloid- β (A β) or *E. coli*, and showed a diminished transcriptional response elicited by myelin. Together, these findings suggest that GPR34 is important for maintaining microglia in a homeostatic state, promotes phagocytosis of and transcriptional response to myelin, and limits microglial activation in neurodegenerative disease conditions.

Introduction

Microglia are resident immune cells of the brain that play a key role in maintaining brain homeostasis and health. They perform diverse functions such as synaptic elimination, debris clearance, immune surveillance, and sculpting neuronal activity and plasticity^{1–7}. Microglial dysfunction is a key feature of neurodegenerative and myelin-related diseases such as Alzheimer's disease (AD) and multiple sclerosis (MS)^{4–6,8,9}. For example, in AD, microglia can be maladaptive, exhibiting changes in gene expression and function such as impaired clearance of myelin debris and amyloid- β , chronic pro-inflammatory responses, and excessive elimination of neuronal synapses. Notably, the expression of many risk genes for AD are highly enriched in microglia^{2,10,11}; however, the signals and mechanisms that regulate the transition of microglia between homeostatic, adaptive, and pathological states remain poorly understood^{1,4,8,9}.

G-protein coupled receptors (GPCRs) mediate various cell-environment interactions and represent the largest set of currently druggable therapeutic targets. Several GPCRs are selectively expressed in microglia, but their functions are poorly understood, despite their potential relevance for therapeutic intervention^{12,13}. One such receptor is GPR34, which is a member of the rhodopsin-like family of GPCRs. *GPR34* is highly and specifically expressed in microglia and macrophages, but its biological role remains largely undefined^{13,14}.

GPR34 expression in microglia is downregulated under various disease conditions such as AD and MS^{14,15}. Further, GPR34 is reported to play a role in maintaining microglial homeostasis, regulating lipid responses, and modulating microglia states and disease pathology^{16–23}. However, the identity of its endogenous ligand(s) remains uncertain; lysoPS has been proposed as a candidate ligand, but because it activates multiple receptors, its biological effects cannot be specifically attributed to GPR34^{24–28}. Studies report conflicting functions for GPR34: some protective, others detrimental against AD pathology^{16,17,19,21–23,29}. Several reports show that GPR34 inhibition alleviates neuroinflammation and promotes amyloid clearance in mice^{17,21,22}, whereas another reported that GPR34 agonism can enhance *in vivo* amyloid clearance¹⁶. These discrepancies highlight the limited understanding of the upstream signals and downstream pathways regulating GPR34 activity in microglia, as well as its **context-dependent functions**.

To address the *in vivo* function of GPR34, we first studied transcriptomic changes in *Gpr34* KO mouse brains by RNA sequencing (RNA-seq). To investigate GPR34's role in an Alzheimer's disease model, we studied the brain and microglia of *Gpr34* KO mice crossed to the 5xFAD mouse model of pathology^{30,31}. Human iPSC-derived induced microglia-like cells (iMGLs) null for *GPR34* were generated and used to dissect downstream signaling of GPR34 in response to various ligands, and to evaluate the resulting effects on gene expression and phagocytosis. We find that *Gpr34* deletion in mice shifts microglia toward inflammation- and DAM-related gene expression signatures, with some resemblance to 5xFAD microglia. In 5xFAD mice, *Gpr34* KO exacerbates the disease-associated features, including heightened glial reactivity. In iMGLs, *GPR34* deletion abolishes downstream signaling in response to both pharmacological agonists and myelin, and diminishes functional responses, including myelin-induced transcriptional effects and phagocytosis. Thus, by utilizing both *in vivo* and *in vitro* models, we were able to clarify how GPR34 influences microglial transcriptional states, signaling, and function in both normal physiologic and chronic disease settings.

Results

Gpr34 knockout in mice shifts microglial transcriptional profiles toward DAM- and inflammation-associated signatures

GPR34 is expressed highly and selectively in microglia within the brain in humans and mice (Supplemental Figure 1 [13,14](#)). To determine how GPR34 loss affects microglial transcriptional programs, we performed single-cell RNA sequencing (scRNA-seq) of isolated microglia and brain border-associated macrophages (BAMs) from hippocampal tissue from 6-month-old wild-type (WT) and *Gpr34* KO mice, on either a normal C57BL/6 background or in 5xFAD transgenic mice, resulting in 4 conditions: WT, *Gpr34* KO (which refers to *Gpr34* ^{-/-}, as *Gpr34* gene is X-linked), 5xFAD, and *Gpr34* KO;5xFAD (Figure 1A-B [15](#); see Methods [16](#)). We also performed single-nucleus RNA sequencing (snRNA-seq) of hippocampal tissue from the same mice (contralateral hemisphere) to study the effect of GPR34 loss on the non-microglial cells. Clustering, quality control, and cell type annotation were performed on both the single-cell and single-nucleus data (Figure 1B [17](#), Supplemental Figure 2 and 3 [18](#)). After standard quality control across samples of the microglia scRNA-seq data, we identified distinct subpopulations as described from previous studies^{9,32}, including a homeostatic state, DAM (which, more specifically, Amyloid-DAMs, as associated with amyloid-disease in 5xFAD mice)¹, interferon-responsive microglia, and a *Ms4a7*⁺ macrophage population representing brain-border associated macrophages¹, and cycling microglia (Supplemental Figure 3A-B [19](#)). We then first focused on the comparison between WT and *Gpr34* KO mice to define the transcriptional consequences of *Gpr34* loss in the absence of 5xFAD pathology.

In *Gpr34* KO mice, we verified data quality across samples (Supplemental Figure 2B [20](#) and Supplemental Table 1 [21](#)) and confirmed the absence of *Gpr34* transcripts in *Gpr34* KO microglia (Supplemental Figure 3C [22](#)). Differential expression analysis of scRNA-seq from all isolated microglia comparing *Gpr34* KO to WT revealed 60 differentially expressed genes (DEGs) with an FDR cutoff of 0.05 (Figure 1C [23](#) and Supplemental Table 2 [24](#)), with many of the DEGs persisting even when performing differential expression analysis between *Gpr34* KO and WT mice within a given subtype of microglia (Supplemental Table 2 [25](#)). Among the DEGs from the analysis with cells from all microglial subtypes (Figure 1C and 1D [26](#)), we observed upregulation of known DAM and inflammatory genes, such as *Anxa5*, *Axl*, *Itgax*, *Cst7*, *Cadm1*, and *Rftn1*. Gene set enrichment analyses (GSEA) of *Gpr34*-deficient microglia revealed transcriptional changes across immune, Alzheimer's disease-related, myelination, cell death, and cholesterol metabolism related modules (Figure 1E-F [27](#)). DAM-related gene sets (more specifically Amyloid-DAM gene set) based on previously published data^{1,9} (see Methods for details) were significantly enriched among upregulated genes of *Gpr34* KO microglia, including genes such as *Spp1* and *Cst7*, while “homeostatic” microglia gene sets were enriched among the downregulated genes (Figure 1E [28](#)). Consistent with these changes, *Gpr34* KO microglia showed increased enrichment of immune and inflammatory gene sets among upregulated genes, particularly cytokine and chemokine signaling, complement activation, and responses to lipopolysaccharide and interferon gamma, along with modest enrichment of Alzheimer's disease-related genes (Figure 1E [29](#)). Functional modules associated with microglial activity such as response to lipids, myelination, cell killing, and GPCR pathways were also enriched among upregulated genes (Figure 1F [30](#)), suggesting altered lipid handling and signaling capacity.

Quantitative analysis of subcluster proportions in the microglia single cell data (Figure 1G [31](#)) revealed a slight but significant reduction of homeostatic microglia and a corresponding expansion of DAM microglia in *Gpr34* KO mice, with other subclasses not significantly changed.

Additionally, we used snRNA-seq to investigate the impacts of *Gpr34* loss on other cell types in the hippocampus (Supplemental Figure 4A [32](#)). Cell type composition analysis indicated a significant increase in the proportion of astrocytes that are disease-associated astrocytes (expressing previously defined markers genes such as *Osmr1*, *Ggta1*, and *Gsn*) and an increase (not reaching statistical significance) in the proportions of disease-associated microglia (DAMs; p-value=.083)

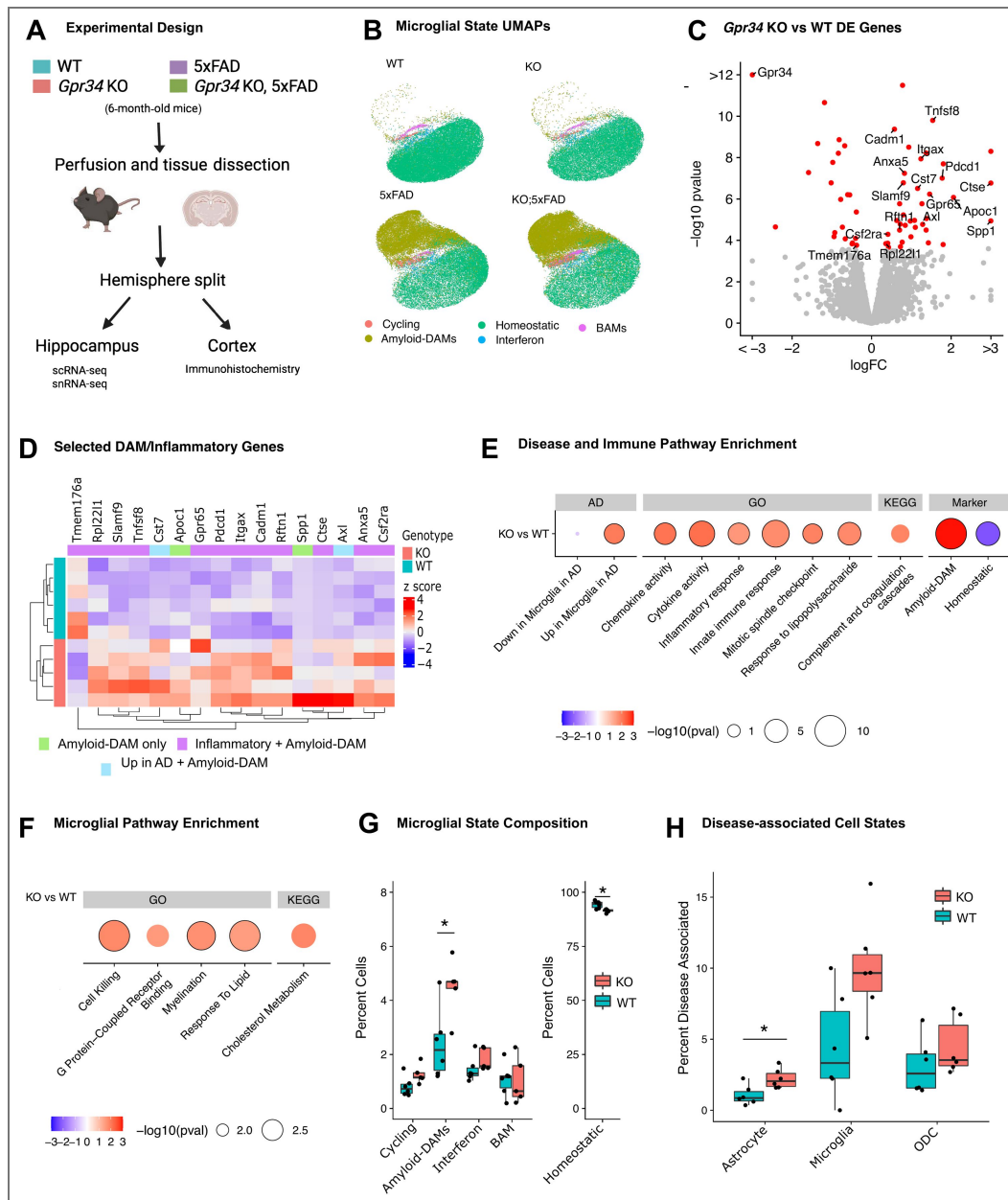


Figure 1. *Gpr34* knockout in mice shifts microglial transcriptional profiles toward DAM- and inflammation-associated signatures, partially resembling 5xFAD Alzheimer's model transcriptomic profile

A: Overview of the experimental design. **B:** UMAP of single-cell microglia data, colored by microglial states. Each condition (*Gpr34* KO, WT, 5xFAD, and *Gpr34* KO:5xFAD) is shown separately. **C:** Volcano plot of differentially expressed (DE) genes between *Gpr34* KO and WT mice in the single-cell data, calculated with pseudobulk. Those colored red are significant. Labeled genes are known to be DAM specific based on previous publications. **D:** Heatmap of selected DE genes. The rows are mice, columns are genes, the color is the z score of the pseudobulked TPM per mouse. The bars on the left represent *Gpr34* KO vs WT, the bars on the top represent which gene set each gene belongs to. All are significant at an FDR of 0.05. **E:** Enrichment analysis of chosen terms related to disease and the immune processes. The terms (see Methods) are on the x-axis, color is Normalized Enrichment Score (NES), size is $-\log_{10} p\text{value}$, those outlined in black have FDR<0.1. The dataset of origin for each term is annotated on the top. **F:** Enrichment analysis of chosen terms related to microglia function. The terms are on the x-axis, color is NES, size is $-\log_{10} p\text{value}$, those outlined in black have FDR<0.1. The dataset of origin for each term is annotated on the top. **G:** Percent of cells (y-axis) in each microglia cell type (x-axis) in each *Gpr34* KO and WT mouse (color). There is a significant (FDR<0.05) reduction in homeostatic cells and increase in DAMs in the *Gpr34* KO mice, represented by the asterisks. **H:** Percent of disease associated cells (y-axis) in astrocyte, microglia and oligodendrocyte (ODC) populations (x-axis) in each KO and WT mouse (color). We see an increase in all of them, though it only reaches significance in the astrocytes (FDR<0.05), represented by the asterisk. **Sample sizes for sequencing analyses:** For single-cell RNA-seq, WT, n = 6 mice; *Gpr34* KO, n = 5 mice; and 5xFAD, n = 5 mice. For single-nucleus RNA-seq, n = 6 mice per condition.

and disease-associated oligodendrocytes (with previously reported markers such as *C4b* and *Serpina3n*; p -value=.29) (Figure 1H [33–35](#)). Notably, these changes occur despite the absence of *Gpr34* expression in non-microglial cells, suggesting that the cell state changes in astrocytes and oligodendrocytes (ODCs) are secondary to the loss of *Gpr34* in microglia. No significant changes in neuronal abundance or other major cell classes were observed, though we were able to identify differentially expressed (DE) genes in numerous cell types, most notably *C4b* in ODCs (Supplemental Figure 4B-G [36](#) and Supplementary Table 3 [37](#)). Note, unlike in the single-cell data, we do not see any DEGs in the microglia in the single-nucleus data. This is likely due to the rarity of microglia in the single nucleus data, leading to low power to detect DEGs.^{7,36}

Together, the transcriptomics data demonstrate that the deletion of *Gpr34* results in a small but significant shift in microglia from a homeostatic state towards a more DAM-like and inflammatory state, correlated with an expansion of DAM proportions and accompanied by (presumably secondary) transcriptomic changes in astrocytes and ODCs. These findings suggest that GPR34 functions to maintain microglia in a homeostatic state *in vivo* and that loss of *Gpr34* may prime microglia toward activation, inflammation, and disease-like states.

***Gpr34* KO microglia partially resemble microglia of the 5xFAD Alzheimer’s model in transcriptomic profile**

To further evaluate the relationship between GPR34 loss and AD-related microglial transcriptomic profiles, we directly compared DE results of *Gpr34* KO vs WT microglia to those for the 5xFAD mouse model of AD vs WT at 6 months. 5xFAD is a well-established mouse model for amyloidosis that exhibits early and aggressive β 1Zamyloid accumulation and robust microgliosis³¹. The original characterization of DAM and homeostatic transcriptional programs of microglia came from a study of the 5xFAD mouse line.⁹ Changes in gene expression in *Gpr34* KO microglia were positively correlated with those observed in 5xFAD microglia. 26 of the 60 genes that were significantly altered in *Gpr34* KO vs WT microglia (Figure 1C [38](#)) were also significantly altered in 5xFAD vs WT microglia, including many upregulated genes (*Cst7*, *Itgax*, *Anxa5*) and downregulated genes (including *Gimap1*, *Gimap6*, and *Gimap9*) all changing in the same direction (20 upregulated genes, 6 downregulated genes) (Figure 2A [39](#)). Directly comparing the long fold changes (logFCs) from the DE analysis (Figure 2B [40](#)) demonstrated positive correlation across the datasets ($R = 0.53$ among genes that reach significance in either comparison), particularly for DAM-associated genes such as *ApoE*, *Spp1*, *Cst7*, and *Axl*. Although well correlated between *Gpr34* KO and 5xFAD, the effect size (log₂FC) of the transcriptomic changes was generally larger for the DEGs in 5xFAD than in *Gpr34* KO microglia (Figure 2B [41](#)). Since both DE gene analyses used the same set of WT samples as controls, overestimation of correlation values can occur. To address this, we applied a permutation-based approach which found the correlation to be significant, confirming that the observed correlation was not driven artifactually by shared controls. As expected, analysis of scRNA-seq from purified microglia showed a reduction in *Gpr34* expression in 5xFAD mice (Figure 2C [42](#)), reaching significance based on the raw p -values reported by EdgeR³⁷ but not significant after applying $fdrtools$ ³⁸ as described in the methods.

***Gpr34* deletion potentiates DAM and inflammatory programs in the 5xFAD Alzheimer’s model**

Given that *Gpr34* KO by itself induces DAM-like and inflammation-related transcriptomic changes that partially overlap with that of 5xFAD microglia, we hypothesized that *Gpr34* KO would further exacerbate the microglial DAM phenotype in the 5xFAD mouse model of amyloidosis. To address this, we crossed *Gpr34* KO mice with 5xFAD mice and performed scRNA-seq of isolated microglia and snRNA-seq of hippocampal brain tissue from 6-month-old animals to assess gene expression and cell-type-specific changes across genotypes (Figure 1A [43](#)).

Comparing *Gpr34* KO;5xFAD to *Gpr34* WT; 5xFAD by scRNA-seq revealed amplification of DAM and inflammatory-associated transcriptional responses in the *Gpr34*-deficient 5xFAD microglia. Differential expression analysis (Figure 2D [44](#)) identified 27 DEGs that were upregulated in *Gpr34*

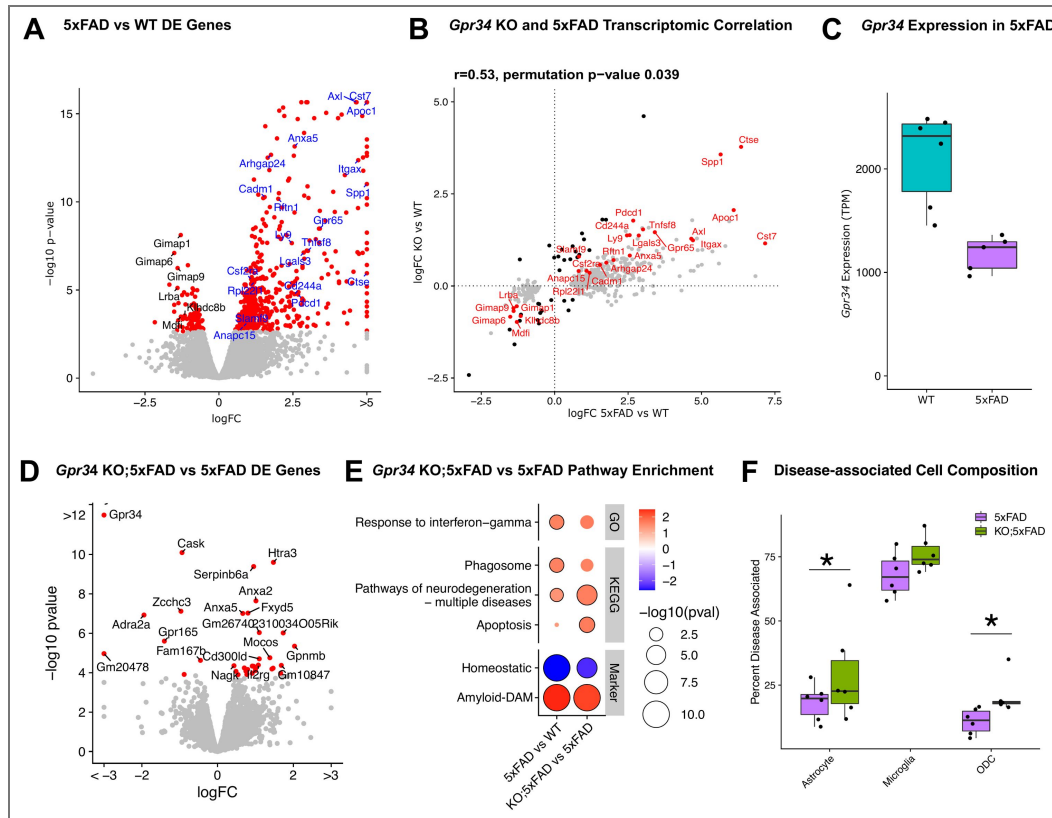


Figure 2. *Gpr34* deletion partially resembles 5xFAD Alzheimer's model transcriptomic profile and potentiates DAM and inflammatory programs in the 5xFAD Alzheimer's model.

A: Volcano plot of DE genes for 5xFAD vs WT microglia in the single-cell data; those colored red are significant. Those gene names colored blue are upregulated in *Gpr34* KO vs WT mice and those in black are downregulated. **B:** Correlation between the logFC of genes in 5xFAD vs WT (x-axis) versus those in *Gpr34* KO vs WT (y-axis) in the single-cell data. We only show those genes that are significant in at least one comparison, the color of each dot represents which comparison each gene is significant in—black dots are significant in KO vs WT, grey dots in 5xFAD vs WT, and red are significant in both. Those that are significant in both are labelled. The correlation and the associated permutation-based p-value (the p-value is calculated so as to overcome the issue of shared controls which can lead to inflated correlations) are shown at the top. **C:** *Gpr34* expression for each mouse (y-axis, measured in TPM) in WT vs 5xFAD microglia (x-axis). **D:** Volcano plot of DE genes in KO;5xFAD vs 5xFAD mice in the single-cell data, calculated with a pseudobulk based method. Those colored red are significant. Those labelled genes are the top 20 most significant genes. **E:** Enrichment analysis of chosen terms related to disease, the immune system and microglia function in the single cell data. The terms are on the y-axis, color is NES, size is $-\log_{10}$ pvalue, those outlined in black have FDR<0.1. The comparison is on the x-axis, either 5xFAD vs WT or KO;5xFAD vs 5xFAD. The dataset of origin for each term is annotated on the right side. **F:** Percent of disease associated cells (y-axis) in each *Gpr34* KO;5xFAD and 5xFAD mouse (color) for each cell type (x-axis) in the single-nucleus data. We see an increase in all of them, though it only reaches significance in the astrocytes and oligodendrocytes (FDR<0.05), represented by the asterisks. **Sample sizes for sequencing analyses:** For single-cell RNA-seq, WT, n = 6 mice; *Gpr34* KO, n = 5 mice; and 5xFAD, n = 5 mice. For single-nucleus RNA-seq, n = 6 mice per condition.

KO;5xFAD microglia relative to 5xFAD microglia, including *Anxa5* and *Gpnmb*, reflecting an exacerbation of the DAM gene signature. There were only 8 significantly downregulated genes comparing *Gpr34* KO;5xFAD to *Gpr34* WT; 5xFAD microglia. Gene set enrichment analysis (Figure 2E) showed enrichment among upregulated genes of immune and microglial functional pathways, apoptosis-related terms, and gene sets associated with DAM and neurodegeneration, along with further downregulation of homeostatic gene sets. Cell-type composition analysis showed an increased proportion of DAMs in *Gpr34* KO;5xFAD microglia relative to 5xFAD, though this did not reach significance (Supplemental Figure 5A).

snRNAseq of hippocampal tissues (Figure 2F) confirmed that *Gpr34* deletion in the 5xFAD model significantly increased the proportion of disease-associated astrocytes and disease-associated oligodendrocytes with a non-significant increase in DAMs^{33–35}. As with the *Gpr34* KO vs WT analysis, we were able to identify many DEGs in various cell types other than microglia (Supplemental Figure 5B–F and Supplemental Table 3).

Taken together, *Gpr34* loss alone induces DAM and inflammation signatures in microglia (Figure 1) and its deletion in 5xFAD mice further amplifies, rather than reverses, the DAM and inflammatory transcriptomic profiles of microglia in 6-month-old 5xFAD mice (Figure 2). These findings suggest that GPR34 plays a modulatory role, restraining disease-associated and inflammatory pathways in response to β IZamyloid pathology.

***Gpr34* loss exacerbates microglial reactivity and increases amyloid beta engulfment in 5xFAD mice**

We examined the effects of *Gpr34* knockout on other aspects of AD pathology (amyloid-beta ($A\beta$) and glial activation) by immunohistochemistry (IHC) of cortical sections of 6-month-old 5xFAD mice with or without *Gpr34* (*Gpr34* KO;5xFAD). Using confocal z-stack imaging and Imaris 3D surface analysis, we observed a non-significant increase in total $A\beta$ -positive burden in the cortical ROIs in *Gpr34* KO;5xFAD mice compared to 5xFAD (Figure 3A and 3B). The total Iba1+ immunoreactivity within cortical ROIs was unaltered between these genotypes (Figure 3C). However, we found that the CD68 signal (a marker of lysosome/phagocytic activity) within Iba1+ microglial compartments, normalized to total Iba1 signal, was significantly increased in *Gpr34* KO;5xFAD mice (Figure 3D–E). Notably, $A\beta$ signal within CD68+ structures (phagosomes/lysosomes) inside Iba1+ microglia was significantly increased in *Gpr34* KO;5xFAD mice (Figure 3F). We also measured GFAP (a marker of astrocyte activation) in the cortex whole-slide immunostaining and 2D image analysis. We found a 45.7% increase in GFAP+ area in *Gpr34* KO;5xFAD mice relative to 5xFAD, although that did not reach significance ($p = 0.08$) (Figure 3G and 3H). Together, these immunohistochemical findings align with our transcriptomic results and suggest that *Gpr34* loss in 5xFAD mice enhances microglial reactivity, amyloid beta engulfment, with a trend towards increased astrocyte reactivity.

Generation and pharmacological validation of *GPR34*-deficient human iPSC-derived microglia (iMGLs)

We next sought to establish a robust platform for mechanistic and pharmacological studies at the cellular level. However, cultured primary murine microglia were inappropriate for these purposes, as *Gpr34* expression is swiftly downregulated upon removal from the CNS^{12,39} (data not shown). We therefore utilized human iPSC-derived microglia-like cells (iMGLs), following a previously established 40-day cytokine-based protocol^{32,40}. We used a CRISPR/Cas9 strategy to introduce precise 2-base-pair deletions into the *GPR34* coding sequence of WTC-11 iPSCs and confirmed an indel mutation in two different *GPR34* KO clones (designated KO-1 and KO-2) by Sanger sequencing and RNA-sequencing (Figure 4A; Supplemental Figure 6A). For each KO clone, a matched isogenic control clone (WT-1 and WT-2) that underwent the identical CRISPR editing and clonal expansion workflow but retained the wild-type *GPR34* sequence was used as a paired control. After differentiation into iMGLs, both KO clones responded similarly to a known *GPR34* agonist, Compound 4B (Supplemental Figure 9A)⁴¹, so we focused on one pair (KO-1 and

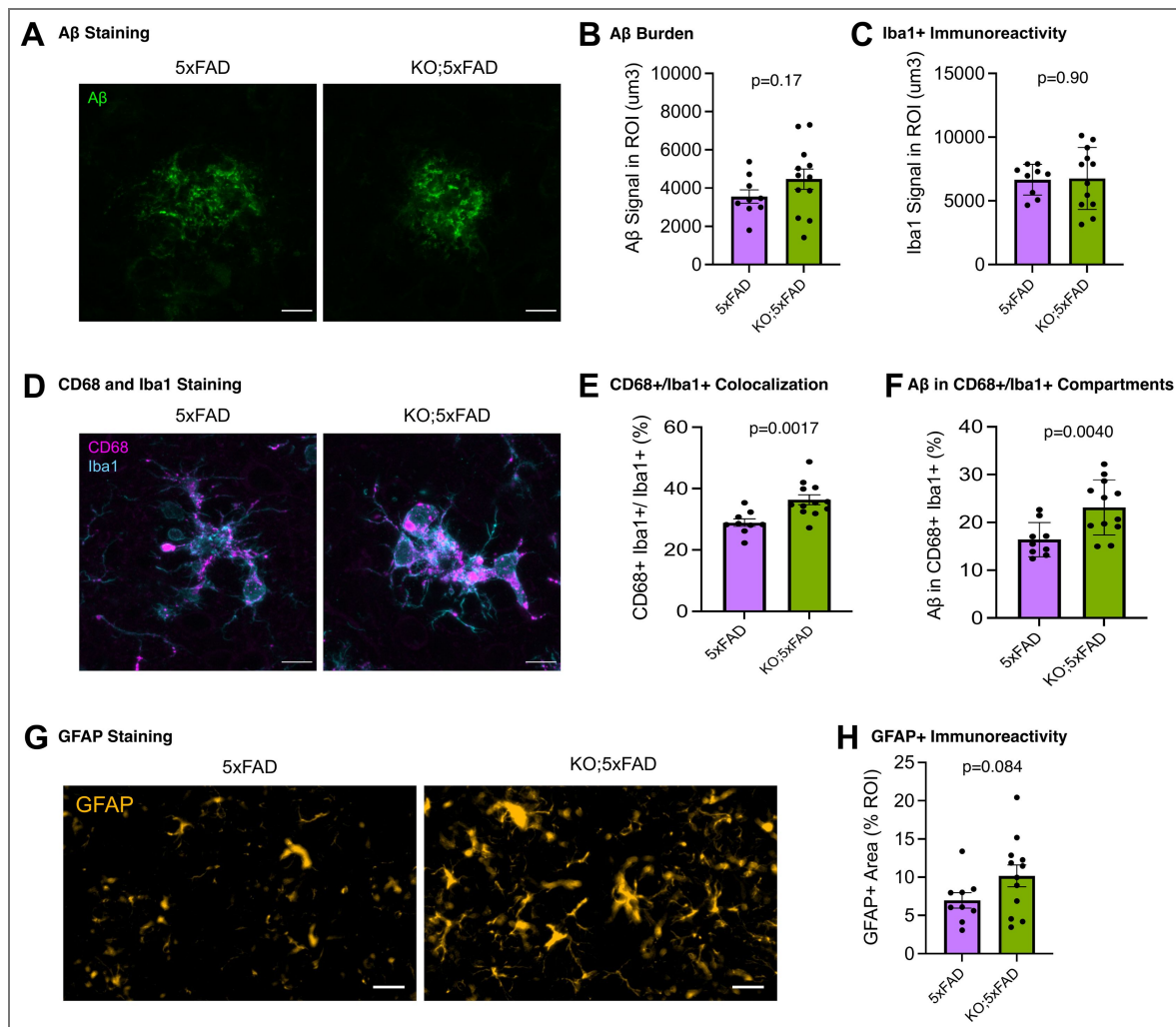


Figure 3. *Gpr34* deletion exacerbates microglial reactivity and increases amyloid beta engulfment in 5xFAD mice

A: Representative image of Aβ (shown in green) immunofluorescence staining in the cortex of 5xFAD and *Gpr34* KO;5xFAD mice. Scale bar indicates 10μm. **B:** Quantification of total Aβ signal in the region of interest (ROI) of the cortex of 5xFAD and *Gpr34* KO;5xFAD mice using confocal z-stack imaging and Imaris 3D surface analysis. Values represent summed Aβ-positive signal within each ROI. P = 0.17. **C:** Quantification of total Iba1 immunoreactivity in the ROI of the cortex of 5xFAD and *Gpr34* KO;5xFAD mice using confocal z-stack imaging and Imaris 3D surface analysis. P = 0.90. **D:** Representative image of Iba1 (shown in cyan) and CD68 (shown in magenta) immunofluorescence staining in the cortex of 5xFAD and *Gpr34* KO;5xFAD mice. Scale bar indicates 10μm. **E:** Quantification of CD68 signal within Iba1+ microglial compartments, normalized to total Iba1 signal, in cortical ROIs from 5xFAD and *Gpr34* KO;5xFAD mice using Imaris 3D surface analysis. Values are normalized to total Iba1 signal. P = 0.0017. **F:** Quantification of Aβ signal within CD68+/Iba1+ compartments in cortical ROIs using Imaris 3D surface analysis. Values are normalized to total Aβ signal. P = 0.0040. **G:** Representative whole-slide immunofluorescence image of GFAP (shown in yellow) staining in the cortex of 5xFAD and *Gpr34* KO;5xFAD mice. Scale bar indicates 20μm. **H:** Quantification of GFAP-positive immunoreactivity within cortical ROIs from 5xFAD and *Gpr34* KO;5xFAD mice, using 2D whole-slide image analysis, measured as percent area. P = 0.084. **Imaging quantification:** 5xFAD, N=9 mice and *Gpr34* KO;5xFAD, N=12 mice. 4-5 brain slices were analyzed for each animal for Aβ, Iba1 and CD68 measurements. 2-3 brain slices were analyzed for each animal for GFAP measurement. Data represent mean ± s.e.m. Welch's t test. **Sample sizes for sequencing analyses:** For single-cell RNA-seq, 5xFAD, n = 5 mice, and *Gpr34* KO;5xFAD, n = 6 mice. For single-nucleus RNA-seq, n = 6 mice per condition.

WT-1) for subsequent experiments. *GPR34* KO-1 iMGLs displayed ~2-fold elevated *GPR34* transcript level relative to WT-1 iMGLs (Supplemental Figure 6B [42](#)). However, quantitative mass spectrometry showed that *GPR34* KO-1 iMGLs indeed lacked detectable GPR34 protein (Figure 4B [43](#)).

To evaluate any gross morphological effects of *GPR34* loss, we performed immunostaining on iMGLs that were dissociated on day 42 and replated at equal cell density. Nuclear staining confirmed comparable cell numbers across all wells and genotypes (Supplemental Figures 7A-B [42,43](#)). Cell area was measured by immunostaining using a panel of cell-surface and intracellular microglial markers consisting of: P2RY12, IBA1, C1q, C3aR, and CD45. All markers exhibited robust staining, validating that these were fully differentiated iMGLs (Supplemental Figure 7A [42](#)). *GPR34* KO-1 iMGLs showed similar immunostaining intensities for all tested markers and no obvious differences in cell morphology were observed between KO and WT iMGLs (Supplemental Figures 7C-D [42](#)). Additionally, flow cytometry conducted on Day 40, upon completion of the microglial differentiation protocol, showed comparable expression of CD45, CD11b, P2RY12, and CX3CR1 microglial markers in WT-1 and *GPR34* KO-1 cells. (Supplemental Figures 8A-B [42](#)).

To determine the functional consequences of *GPR34* loss on downstream pathways and ligand responses, we used a set of pharmacologically validated tool compounds in our iMGL platform and examined downstream GPCR signaling pathways associated with GPR34⁴⁴ (Figure 4C [44](#)). GPR34 activation by a validated agonist, Compound 4B, elevated intracellular calcium levels with an EC₅₀ of approximately 3 nM (Figure 4D [44](#) and Supplemental Figure 9A [44](#)) and increased ERK1/2 phosphorylation with an EC₅₀ of approximately 0.5 nM (Figure 4E [44](#)). Both responses were abolished in *GPR34* knockout iMGLs (Figure 4D-E [44](#)) and by the GPR34 antagonist YL-365⁴⁵ (Supplement Figure 9B-C [44](#)). GPR34 is thought to be a G_{ai}-coupled receptor⁴⁴. However, upon treatment with Compound 4B, the level of forskolin-induced cAMP was unchanged, suggesting that GPR34 does not significantly modulate cAMP signaling under these conditions (Supplemental Figure 9D [44](#)). These data validate our iMGL platform for assessing GPCR signaling, demonstrating robust and receptor-dependent activation of canonical downstream signaling pathways.

Lysophosphatidylserine (LysoPS), which has been proposed as an endogenous GPR34 ligand, stimulated calcium and pERK responses in wildtype iMGLs¹⁷. Responses to LysoPS were reduced in the *GPR34* knockout iMGLs, albeit incompletely and with a rightward shift in the dose response curve (Figure 4F-G [44](#)), suggesting the presence of additional LysoPS-responsive receptors in iMGLs besides GPR34. Myelin debris, which is lysoPS-rich, has recently been reported to promote microglial dysfunction, neuroinflammation, and AD-related pathology via GPR34^{19,22}. Consistent with this, we found that myelin also induced calcium elevations in microglia, which was blocked in *GPR34* KO-1 iMGLs (Figure 4H-I [44](#)), or by a GPR34 antagonist, (S)-E49 (Figure 4H [44](#)) ((S)-E49 - Itoh et. al. Patent application publication US2010/0130737). By contrast, calcium signals elicited by agonists of other microglia GPCRs (P2Y12 and C3aR), remained largely intact in *GPR34* KO-1 iMGLs (Figure 4J-K [44](#)).

Together, these findings show that GPR34 primarily regulates calcium and pERK signaling but not cAMP in human iPSC-derived microglia and that LysoPS, the proposed endogenous ligand, acts, at least in part, in a GPR34-dependent manner. Importantly, myelin debris also stimulates calcium signaling in microglia in a GPR34-dependent manner.

***GPR34* deficiency selectively impairs myelin phagocytosis in iPSC-derived microglia**

Phagocytosis is a core microglial function essential for homeostatic maintenance of the central nervous system (CNS)^{2,5,6}. Our data indicate that myelin, a key *in vivo* phagocytic substrate, can signal through GPR34 (See Figure 4H-I [44](#)), leading us to examine whether GPR34's interaction with myelin also regulates its engulfment. To directly investigate the role of GPR34 in this fundamental process, we performed *in vitro* phagocytosis assays using iMGLs from WT-1 and *GPR34* KO-1

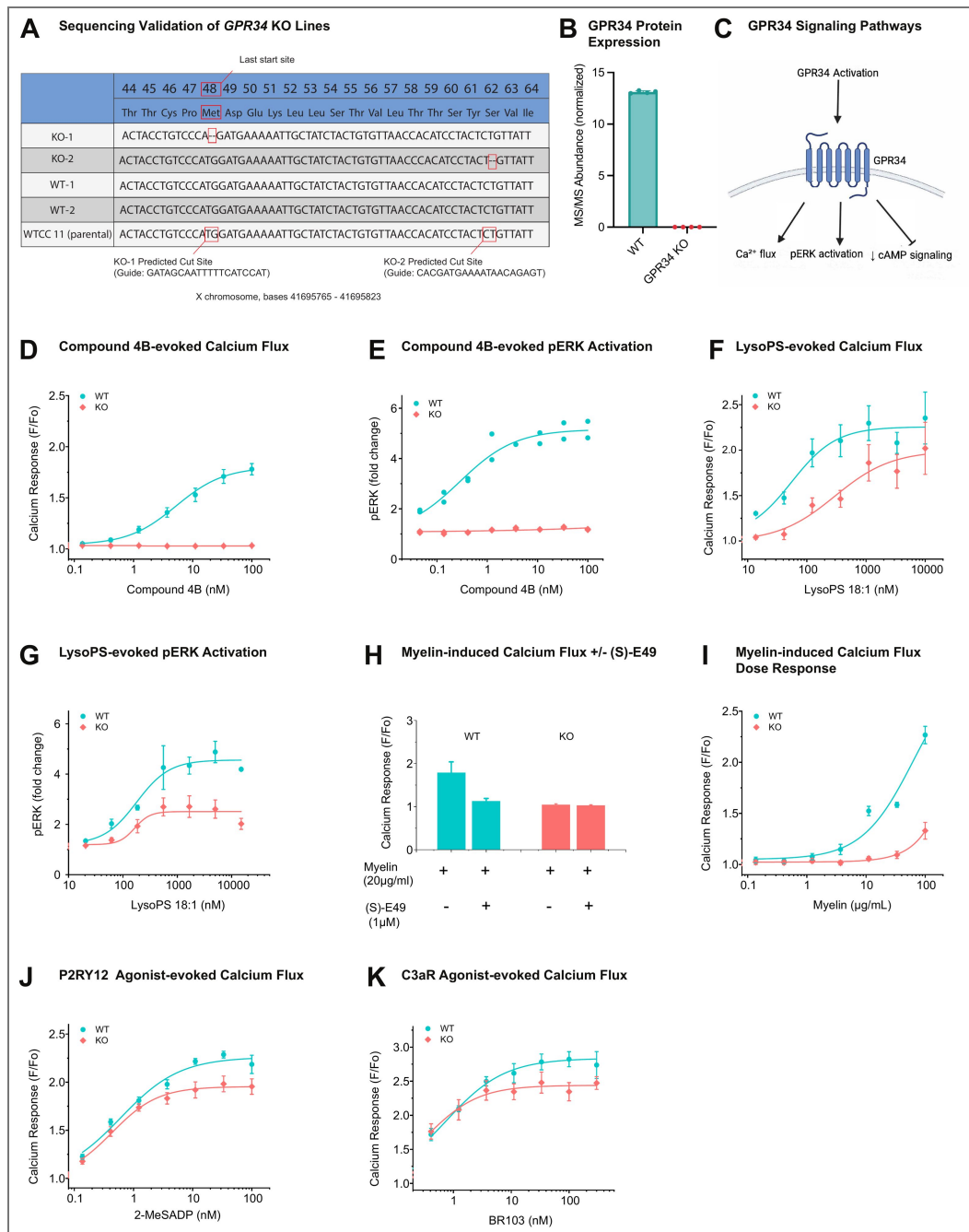


Figure 4. Generation and pharmacological validation of *GPR34*-deficient human iPSC-derived microglia (iMGLs)

A: Sequencing validation of two independent *GPR34* iPSC knockout (KO) clones (KO-1 and KO-2) and their respective WT controls. Guide RNA sequences for KO-1 and KO-2 are indicated below the alignment, with predicted Cas9 cut sites marked. Red boxes indicate cut sites, confirming editing of *GPR34* in the KO clones relative to WT and parental controls **B:** MS/MS analysis shows the loss of *GPR34* protein in *GPR34* KO-1 iMGLs **C:** Schematic illustrating *GPR34* activation and downstream signaling pathways. **D:** Dose-dependent calcium response to the *GPR34* agonist Compound 4B in WT and *GPR34* KO iMGLs. **E:** Dose-dependent pERK activation in response to the *GPR34* agonist Compound 4B in WT and *GPR34* KO iMGLs. **F:** Dose-dependent calcium response to the *GPR34* endogenous ligand lysoPS in WT and *GPR34* KO iMGLs. The representative curve was shown from the average data from triplicates. **G:** Dose-dependent pERK activation in response to the *GPR34* endogenous ligand lysoPS in WT and *GPR34* KO iMGLs. **H:** Myelin-induced calcium signaling in WT and *GPR34* KO iMGLs treated with (S)-E49. **I:** Dose-response of myelin-induced calcium flux in WT and *GPR34* KO iMGLs. **J-K:** Dose-dependent calcium response to *GPR34* unrelated agonists (P2Y12/2-MeSADP, C3aR/BR103) in WT and *GPR34* KO iMGLs. **Sample sizes and stats:** *GPR34* KO line 1 (KO-1) and its control (WT-1) were used for this study. The representative curve for D, F, G, H and I was shown from the average data are from triplicates and for E is from duplicates.

iMGLs, along with the GPR34 antagonist YL-365⁴⁵. These experiments build on prior findings suggesting that GPR34 contributes to microglial phagocytosis *in vivo*²⁰ and substrate selectivity *in vitro*¹⁶.

We assessed the phagocytic uptake of three substrates: myelin debris, amyloid fibrils, and *E. coli* (Figure 5A⁴⁵, schematic). Each substrate was conjugated to pHrodo Deep Red, a pH-sensitive fluorophore that becomes fluorescent in acidic compartments such as the phagolysosome. After exposure of WT-1 and *GPR34* KO-1 iMGLs to each pHrodo-labeled substrate, we measured median fluorescence intensity (MFI) using two complementary approaches: live confocal imaging 5 and 24 hours after substrate addition (Figure 5A⁴⁵), and flow cytometry at the 25-hour terminal time point (Supplemental Figure 10⁴⁵). Both methods showed a significant 30% reduction of myelin uptake in *GPR34* KO-1 microglia compared to WT-1 cells (95% CI: 12-49%) (Figures 5B⁴⁵ and Supplemental Figures 10A-B⁴⁵). In contrast, phagocytosis of amyloid fibrils and *E. coli* bioparticles was not significantly affected (Figure 5C-D⁴⁵ and Supplemental Figures 10C-D⁴⁵). To test whether pharmacological inhibition of GPR34 could replicate this selective impairment, iMGLs were pre-treated with the GPR34 antagonist YL-365 (10 μ M) for 30 minutes prior to substrate exposure. Antagonist-treated cells showed a similar reduction in myelin phagocytosis, with no effect on uptake of the other substrates (Figures 5E⁴⁵ and Supplemental Figures 10E-H⁴⁵). Together, these results indicate that, at least *in vitro*, GPR34 plays a selective and critical role in microglial phagocytosis of myelin debris.

Multiomic analysis of *GPR34*-deficient iMGLs highlights dysregulation of immune, replicative, and lysosomal programs

To investigate the molecular consequences of GPR34 loss in human iMGLs, we performed bulk RNA sequencing and quantitative mass spectrometry (MS) proteomics on WT-1 versus *GPR34* KO-1 iMGLs. Principal component analysis (PCA) of RNA-seq data demonstrated clear separation of WT-1 and KO-1 iMGLs on PC1 across multiple rounds of differentiation (Supplemental Figure 11A⁴⁵).

Differential expression analysis revealed broad transcriptional differences between *GPR34* KO-1 and WT-1 iMGLs (Figure 6A⁴⁵, Supplement Table 4⁴⁵). Gene set enrichment analysis (GSEA) highlighted enrichment of DAM-related genes, and of pathways related to phagocytosis, endocytosis, and lysosomal function among the upregulated genes, consistent with increased expression of genes such as *PLIN2*, *CD36*, *LAMP2* and *SYT11*^{46–49}. Conversely, pathways associated with DNA replication, cell cycle, and proliferation were downregulated with genes such as *MCM4*, and *TOP2A*, and *CBX5* contributing to these changes^{50–52} (Figure 6B-C⁴⁵).

Consistent with the transcriptomic analysis, PCA analysis of the proteomics dataset demonstrated distinct clustering of WT-1 and KO-1 samples (Supplemental Figure 11B⁴⁵). *GPR34* deletion produced a pronounced effect on the iMGL proteome, with thousands of differentially expressed proteins (DEPs) surpassing the significance threshold (FDR-adjusted $P < 0.05$) (Figure 6D⁴⁵). GSEA indicated enrichment of proteins related to T-cell activation and MHC Class II complex formation (FDR adjusted $P < 0.05$) among the upregulated DEPs, whereas pathways related to the MCM complex, DNA replication initiation, multivesicular body organization, endocytic regulation, and ESCRT I complex were downregulated (Figure 6E⁴⁵).

Within the vesicular trafficking pathways, VPS37B, a core ESCRT component required for endosomal sorting⁵³, was strongly downregulated at both the transcript and protein levels ($\log_2$ FC = -2.77, adjusted $p = 6.8 \times 10^{-15}$ for RNA; $\log_2$ FC = -1.23, adjusted $p = 8.9 \times 10^{-6}$ for protein) (Figure 6A⁴⁵, Figure 6D⁴⁵). The broader proteomic landscape also emphasized endosomal and lysosomal dysfunction, evidenced by reduction of ESCRT complex components (MVB12A, VPS37B, VPS37C, VPS28), lysosomal enzymes (CTSL, CTSB, CTSC, GNS), lysosomal membrane proteins (OSTM1), and regulators of endosomal trafficking (BLOC1S1, FLOT1, VPS13C, DYNLRB1, CLTCL1) (Figure 6F⁴⁵). Notably, these reductions were accompanied by a striking increase in GPNMB at the protein level, a putative biomarker of lysosomal impairment, and a canonical DAM marker^{54,55} (Figure 6F⁴⁵). Together, these data highlight vesicular trafficking and lysosomal biology as microglial processes likely disturbed by GPR34 loss of function.

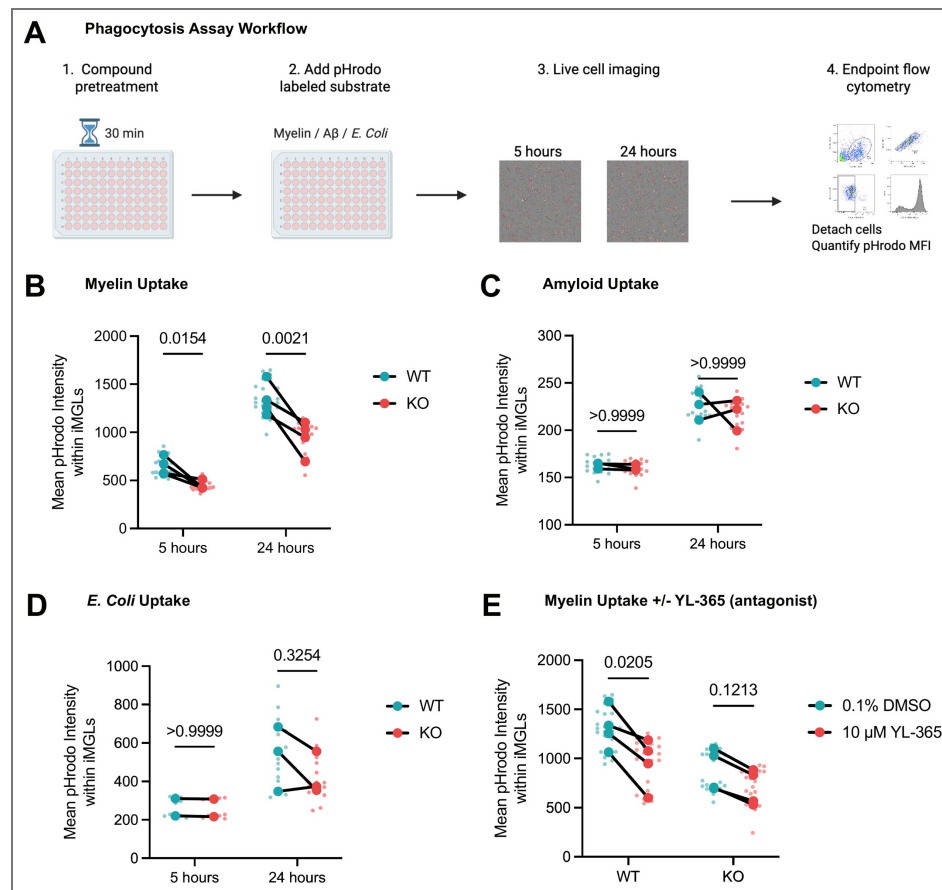


Figure 5. GPR34 deficiency selectively impairs myelin phagocytosis in iPSC-derived microglia

A: Experimental workflow for live-cell imaging and flow cytometry-based assessment of phagocytosis. iMGLs were incubated with pHrodo-labeled substrates and imaged following 5 and 24 hours of substrate incubation using the Opera Phenix live-imaging system. Cells were then detached from imaging plates onto PhenoPlates and processed by flow cytometry on the CytoFLEX LX for assessment of median fluorescence intensity (MFI). **B-D:** WT and *GPR34* KO iMGLs were exposed to pHrodo-labeled myelin (**B**), amyloid fibrils (**C**), or *E. coli* bioparticles (**D**) and assessed by live cell imaging at 5 and 24 hours. Lines connect paired WT and KO biological replicates derived from the same experimental batch. **E:** WT and *GPR34* KO iMGLs were exposed to GPR34 antagonist YL-365 (10 μ M) and assessed for phagocytosis of pHrodo labeled myelin at 24 hours by live cell imaging. **Sample sizes and stats:** Data are represented as mean values with $\pm$ SEM. Smaller circles represent technical replicates, and larger circles represent biological replicates. Statistical tests were undertaken at 5h, 24h, and 25h (n=2-4 independent biological replicates with 5-6 technical replicates per condition). Two-way ANOVA with Bonferroni correction, multiple comparisons test, paired t-test with Welch's correction, as appropriate.

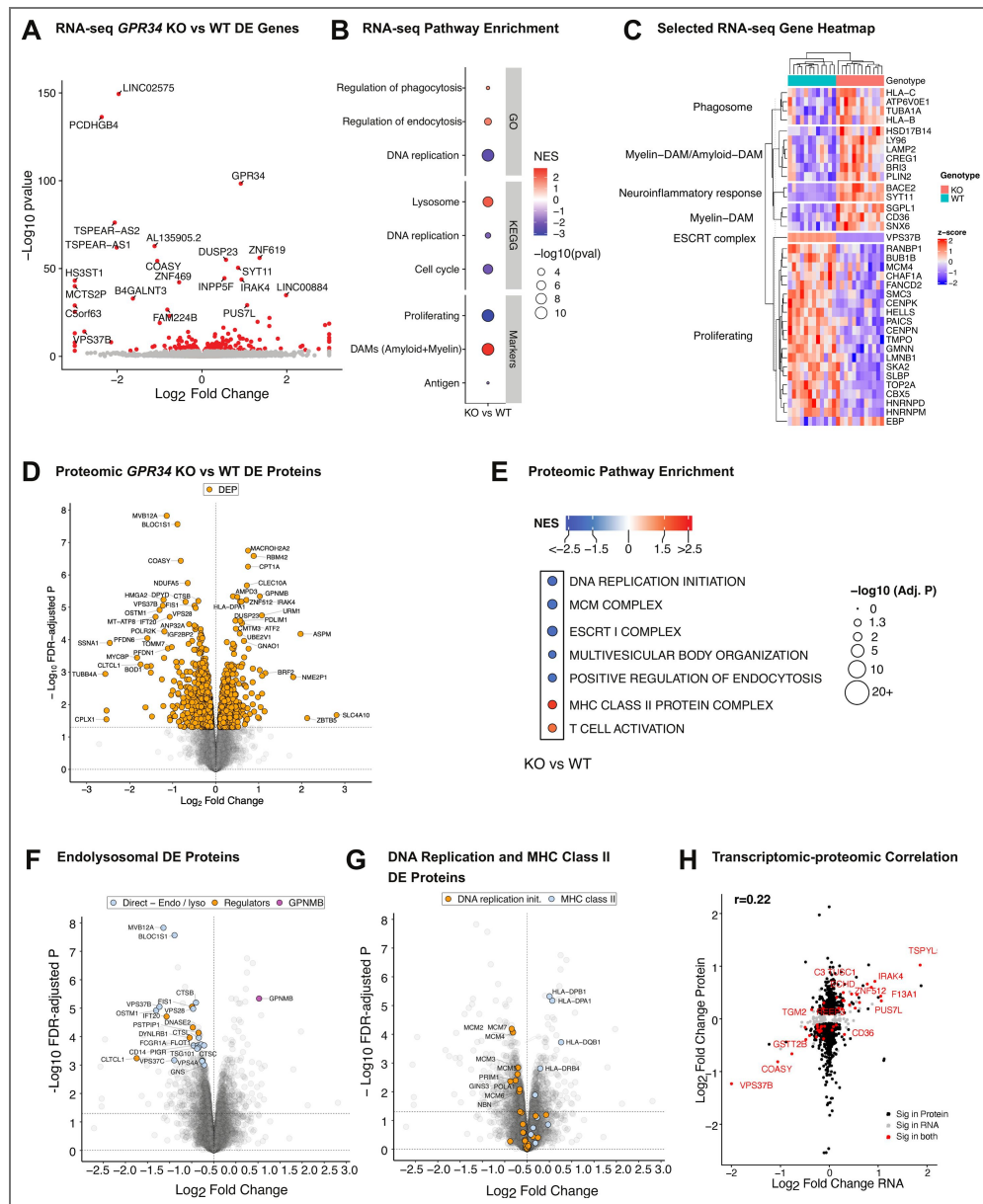


Figure 6. Multiomic analysis of *GPR34*-deficient iMGLs highlights dysregulation of immune, replicative, and lysosomal programs

A: Volcano plot of RNA-seq analysis in *GPR34* KO vs WT iMGL samples. Red dots are significant, top genes (those with smallest p-values) are labelled. Based on the meta-analysis of two experimental batches. **B:** Plot of selected gene sets (y-axis) from gene set enrichment analysis for the *GPR34* KO vs WT RNA-Seq comparison. Color is NES, size is $-\log_{10}$ p-value, those circled in black are significant ($FDR < 0.1$). **C:** Heatmap of selected genes from pathways altered in *GPR34* KO vs WT iMGL samples. The columns are samples, rows are genes, the color is the z score of the TPM expression values. The bars on the top represent KO vs WT. Note the separation between genes from different gene sets (y-axis). **D:** Volcano plot of differentially expressed proteins (DEPs) in MS/MS quantitative proteomics analysis of *GPR34* KO vs WT iMGLs. Top DEPs are labelled. **E:** Gene set enrichment analysis of quantitative proteomics data highlighting prominently changed molecular pathways in *GPR34* KO vs WT iMGLs. Color indicates normalized enrichment scores (NES) and bubble size indicates FDR-adjusted P -value. All shown pathways are significantly altered ($FDR\text{-adjusted } P < 0.05$) in KO iMGLs (compared to WT). **F:** Volcano plot of quantitative proteomics analysis of *GPR34* KO vs. WT iMGLs highlighting endosomal and lysosomal pathways. Proteins directly involved in endosome/lysosome function (“direct - endo/lyso”) and known regulators of these pathways (“regulators”) are indicated. GPNMB, a putative biomarker of lysosomal dysfunction, is also highlighted. **G:** Volcano plot of quantitative proteomics analysis of *GPR34* KO vs WT iMGLs highlighting DNA replication and MHC class II pathways. **H:** Comparison of transcriptomic (x-axis) and proteomic (y-axis) changes ($\log_2 FC$) in *GPR34* KO vs WT iMGLs. Color indicates DE in RNA, protein, or both. Only genes that are DE in either or both assays are shown.

Consistent with the transcriptional downregulation of proliferative pathways in RNAseq data, we observed reduced abundance of DNA replication proteins, including multiple MCM family members (MCM2-7) in *GPR34* KO-1 iMGLs (Figure 6G). These molecular phenotypes suggest reduced proliferation of *GPR34*-deficient cells, which is supported by our findings that KO-1 cultures yielded fewer cells than WT-1 during the iMGL differentiation protocol (Supplemental Figure 11C). In parallel, antigen presentation pathways were strongly upregulated, driven by increased protein levels of MHC class II molecules such as HLA-DPB1, HLA-DPA1, HLA-DQB1, and HLA-DRB4, highlighting a shift from proliferative to immune-related programs^{56–58}.

Finally, integration of transcriptomic and proteomic datasets demonstrated significant albeit modest correlation between respective log₂FCs of RNAs and proteins ($r = 0.22$, $P = 2.96 \times 10^{-17}$) (Figure 6H). In general, the magnitude of change was much greater on the proteome than on the transcriptome, implying that many of the changes driven by loss of *GPR34* occur through post-transcriptional mechanisms, which suggest that proteomics can detect biologically relevant changes that are not present at the mRNA level. Nonetheless, several prominently altered DEGs exhibited directionally consistent and pronounced changes in RNA and protein levels, including downregulation of VPS37B, a core ESCRT-I component required for lysosomal degradation, and COASY, which encodes coenzyme A synthase⁵⁹.

Together, our omics data in human iMGLs indicate that loss of *GPR34* impacts broadly microglial RNA and protein expression, with upregulation of pathways related to immune activation, and suppression of pathways related to DNA replication, phagocytosis, lysosomes and vesicle trafficking.

***GPR34* loss blunts microglial transcriptional responses to myelin challenge**

Myelin is a potent stimulus for microglia, inducing DAM and inflammatory gene expression signatures³². Since *GPR34*-deficient microglia show impaired calcium signaling in response to myelin (Figure 4H-I) and selective deficits in myelin phagocytosis (Figure 5B-E), we next investigated whether *GPR34* alters microglial transcriptional responses to myelin. To capture both early and sustained responses, WT-1 and *GPR34* KO-1 iMGLs were exposed to myelin in culture for 2 hours or 24 hours, followed by bulk RNA sequencing.

Principal component analysis (PCA) revealed that the transcriptome of 24-hour myelin-treated samples clustered distinctly from the 2-hour and untreated (no-myelin) groups, reflecting transcriptional changes induced by prolonged myelin exposure (Figure 7A). The 2-hour and no-myelin samples clustered more closely, consistent with modest early transcriptional responses. In all treatment conditions, WT-1 and KO-1 samples clustered separately from each other, in line with our previous analyses of *GPR34* KO-1 microglia (Figure 7A). Differential expression analysis and transcriptome-wide impact (TWI) scores⁶⁰ from KO-1 vs WT-1 iMGL samples confirmed that myelin induced a substantial transcriptomic shift, with KO-1 samples showing attenuated responses at both time points (231 DEGs in KO-1 vs 921 DEGs in WT-1 at 2 hours, 5648 DE genes in KO-1 vs 8370 genes in WT-1 at 24 hours, (Figure 7B and Supplemental Figure 12A). Despite fewer DEGs in KO-1 samples treated by myelin, many of the DEGs at 24 hours were shared with WT-1 iMGLs treated with myelin (4,729 shared DEGs, $r = 0.89$, based on genes DE at an FDR cutoff of 0.05 in at least one of the two comparisons) (Figure 7C; Supplemental Figure 12B). Overall, the loss of *GPR34* attenuated the broader transcriptomic response induced by prolonged myelin exposure but did not abolish or profoundly alter the nature of the response, implying that additional myelin-responsive pathways act alongside *GPR34*. This contrasts with acute calcium signaling response to myelin, which appears to be largely mediated by *GPR34* (Figure 4H-I).

At 2 hours, myelin induced a significant transcriptional response in WT-1 iMGLs with roughly 1,500 DEGs, characterized by rapid upregulation of immediate-early (IEGs) and cytokine-responsive genes such as *TNF*, *DUSP1*, and *FOS* consistent with acute activation of microglia (Supplemental Figure 12C). Caution must always be used with IEGs since such genes are known to also be related to certain activation artifacts, as has been reported previously⁶¹. By 24 hours, the

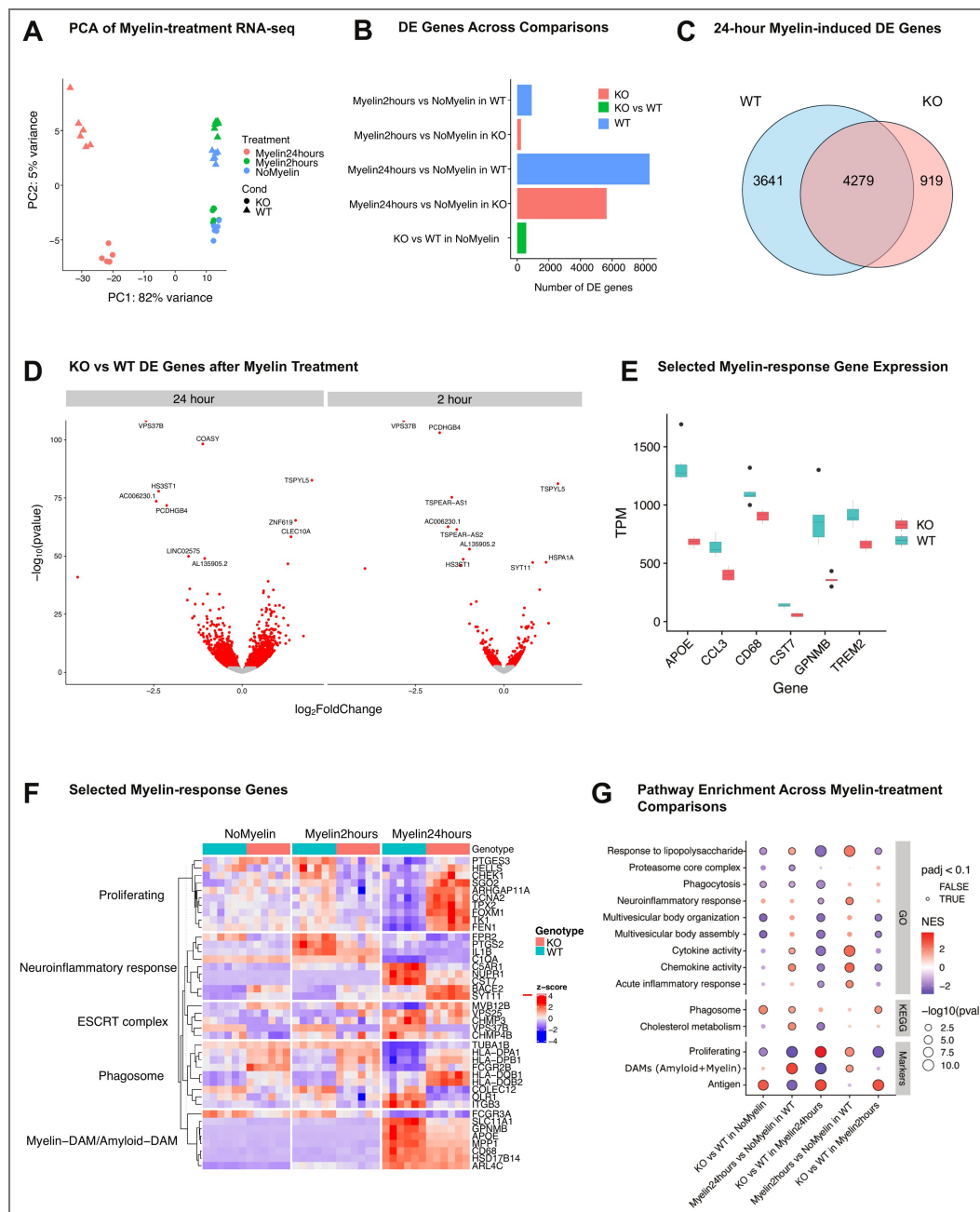


Figure 7. *GPR34* loss blunts microglial transcriptional responses to myelin challenge

A: PCA plot of iMGL bulk RNA-seq samples, colored by myelin treatment. The genotype is represented by the shape. **B:** Bar plots of the number of DE genes (x-axis) for each of the myelin-treatment comparisons (y-axis). **C:** Venn diagram of the number of DE genes after 24-hour myelin treatment versus no-myelin in WT and *GPR34* KO iMGLs. Note overlaps are only counted if they have the same direction of effect. **D:** Volcano plots comparing *GPR34* KO versus WT iMGLs after 24-hour or 2-hour myelin treatment. Significant hits are colored red ($FDR < .05$), genes with the smallest p-values are labelled. **E:** Expression of selected microglial response genes *APOE*, *CCL3*, *CD68*, *CST7*, *GPNMB*, *TREM2* (TPM, y-axis) in WT and *GPR34* KO iMGLs (x-axis) after 24-hour myelin treatment. In the box plots the center line is the median, the boxes span from 25% to 75%, the length of the whiskers is based off the smallest/largest value within 1.5 of the interquartile range from the median, and everything outside that range is plotted as an outlier. **F:** Heatmap of selected myelin-response genes across genotypes and myelin-treatment conditions. The columns are samples, rows are genes, the color is the z score of the TPM expression values. The bars on the top represent KO vs WT. Note the separation between samples with different treatments (x-axis) and genes from different gene sets (y-axis). **G:** Gene set enrichment analysis across genotypes and myelin-treatment comparisons. The terms are on the y-axis, color is NES, size is $-\log_{10}$ p-value, those outlined in black have $FDR < 0.1$. The comparison is on the x-axis. The dataset of origin for each term is annotated on the right side.

transcriptional response to myelin expanded to roughly 8,000 DEGs and shifted toward lipid-related gene sets. Specifically, myelin exposure triggered downregulation of cholesterol biosynthesis genes (*HMGCS1*, *MVK*, *FDFT1*, *DHCR24*), upregulation of LDLR negative regulator (*MYLIP*), downregulation of cholesterol uptake gene (*LDLR*), and upregulation of cholesterol export genes (*ABCG1* and *PLTP*), collectively suggesting changes in handling and export of cholesterol (itself a component of myelin)^{62–65} (Supplemental Figure 12C [↗](#)).

To address whether *GPR34* deficiency alters the microglial response to myelin, we compared the transcriptional changes between WT-1 and KO-1 iMGLs. At 24 hours following myelin treatment, *VPS37B* (a core ESCRT-I component required for lysosomal degradation) and *COASY* (which catalyzes the final steps of coenzyme A biosynthesis) were both reduced in KO-1 iMGLs relative to WT-1 samples (Figure 7D [↗](#)). Relative to untreated controls, *VPS37B* was upregulated by myelin in both genotypes. In contrast, *COASY* was downregulated in WT-1s after myelin treatment but was not significantly different in KO-1 cells. These changes suggest impaired vesicle trafficking and altered metabolic-related programs in KO-1 iMGLs. Upregulation of DAM-related and lysosomal-related genes such as *APOE*, *CCL3*, *CD68*, *CST7*, *GPNUMB*, and *TREM2* was also blunted in KO-1 iMGLs after 24-hour myelin exposure (Figure 7E, F [↗](#), Supplement Table 4 [↗](#))⁹.

At the pathway level, myelin exposure in WT-1 iMGLs at 2 hours led to enrichment among upregulated genes of pathways related to chemokine and cytokine activity, neuroinflammatory response, and proliferation (Figure 7G [↗](#)). In the KO-1 versus WT-1 comparison, many of these immune-related and proliferation pathways were instead downregulated, reflecting a blunted early transcriptional response of KO-1 iMGLs to myelin. By 24 hours, WT-1 samples showed enrichment of upregulated genes in DAM signatures, cholesterol metabolism, and maintenance of immune and inflammatory responses, alongside a concurrent decrease in proliferation. In the KO-1 versus WT-1 comparison, these responses were altered: DAM- and immune-related programs, as well as phagocytosis pathways, were downregulated, while proliferation pathways were upregulated in the KO-1, suggesting an attenuated or dampened transcriptional response relative to WT-1 controls (Figure 7G [↗](#)).

We observed that myelin treatment (2 hours and 24 hours) elicits similar gene-set-level transcriptional responses in WT-1 and KO-1 iMGLs (Supplemental Figure 12D [↗](#); see also Figure 7B, C, G [↗](#) and Supplemental Figure 12B [↗](#)). Altogether, our data suggest that *GPR34* is not required for microglial transcriptomic response to myelin stimulus but may modulate the magnitude of the response. We hypothesize that there are other receptors on microglia, besides *GPR34*, that can recognize myelin or the components of myelin.

Discussion

Although *GPR34* has long been recognized as a microglia-enriched GPCR whose expression is misregulated in various brain disease conditions, its normal physiological functions in microglia and its relevance to human neurodegenerative diseases have remained unclear^{14,18,20,21,26,66,67}. In the mouse brain, loss of *Gpr34* shifted microglia toward DAM and inflammatory-related transcriptional states (Figure 1 [↗](#)). The transcriptional modules upregulated in *Gpr34*-deficient microglia overlapped with those upregulated in the 5xFAD model of AD. *Gpr34* KO in 5xFAD mice further potentiated DAM- and inflammation-related features of microglia and exacerbated glial activation with no change in amyloid burden in this amyloidosis mouse model (Figures 2 [↗](#)–3 [↗](#)). Together these data suggest that *GPR34* plays a role in preventing activation of microglial pathways that are associated with disease and inflammation. Our findings build on previous studies showing that *Gpr34* deficiency increases immune and cytokine gene expression^{18,20} and accelerates transition to DAM microglia²¹. Overall, these data support a **model in which *GPR34* helps maintain microglial homeostasis**, consistent with its high expression in homeostatic microglia and suppression in disease-associated microglia, including in our reanalysis of a published DAM dataset⁹. **The generation of the DAM and homeostatic gene sets is described in the Methods.**

In 5xFAD brains, we found reduced *Gpr34* expression, consistent with reports that *Gpr34* levels are reduced in human brain tissue from neurodegenerative patient contexts¹⁶, although another recent paper reported the opposite trend²². Our RNA-seq and histopathological results from *Gpr34* KO;5xFAD mice appear to contrast with recent reports that *Gpr34* genetic disruption or inhibition with drugs alleviates neuroinflammation and promotes amyloid clearance in the APP/PS1 and 5xFAD models^{17,21,22} and with a separate report in which *Gpr34* agonist treatment promotes *in vivo* clearance of amyloid plaque in the APP/NLGF model¹⁶. These apparent discrepancies may reflect differences in model severity, experimental approach, disease stage, or timing of intervention.

Notably, our study focuses on an earlier timepoint (6 months) in 5xFAD mice compared to 9 months of age in Zhou et al., 2025 when inflammatory pathways in microglia may be differently or more strongly engaged²². Lin et al., 2023 studied *Gpr34* knockdown in the hippocampus of APP/PS1 mice, introducing another layer of variability¹⁷. In addition, our study utilized a constitutive *Gpr34* KO, introducing developmental effects on microglia prior to the appearance of amyloidosis, in contrast to a pharmacological perturbation²² or genetic intervention after the onset of disease pathology^{17,19}. Future systematic studies that compare the temporal and mechanistic dimensions of GPR34 function will be essential to clarify whether, when, and how its functions change during development or disease progression.

GPR34-deficient iMGLs displayed blunted Ca^{2+} and pERK signaling in response to LysoPS-derived agonists and myelin (Figure 4 [C](#)). Our findings align with prior reports showing that GPR34 mediates LysoPS- and myelin-derived signaling and regulates microglial lipid homeostasis^{19,21,22}. Interestingly, while LysoPS-derived agonists of GPR34 such as Compound 4B activated robust signaling in iMGLs, LysoPS itself induced responses that were only partially dependent on GPR34. This suggests that there are additional receptors that can sense Lyso-PS and related lipids^{14,26,28,45,68}. Interestingly, we found that *GPR34*-deficient iMGLs were only deficient in phagocytosis of myelin with no impairment in β -amyloid or *E. coli* uptake. This is in concordance with the idea that myelin uptake involves LysoPS/GPR34 interactions, since synthetic amyloid fibrils do not contain Lyso-PS and bacteria are very low in Lyso-PS. This contrasts with prior studies reporting variable outcomes^{16,19,20,22}. Lin et al., 2024 found no effect of *Gpr34* loss on myelin uptake¹⁹. Etani et al., 2024 reported increased β -amyloid phagocytosis with GPR34 activation without affecting myelin¹⁶, and Preissler 2015 described reduced uptake of zymosan and FBS-coated particles in *Gpr34*-KO mouse microglia²⁰. These discrepancies likely reflect differences in model systems, assay and other experimental factors. Two of these studies evaluated myelin uptake in primary mouse microglia^{16,19}. However, primary mouse microglia rapidly downregulate GPCR expression upon removal from the CNS and during extended culture, unlike physiologically maintained iPSC-derived microglia, in which *GPR34* expression remains relatively stable¹³ (our unpublished data). Further, acute pharmacological activation to interrogate GPR34-dependent uptake¹⁶, may not corroborate with the impacts of long-term genetic loss in a knockout model²⁰.

GPR34 signaling can modulate microglial state and appears to favor a homeostatic rather than inflammatory function of microglia. Therefore, we hypothesize that pharmacologic approaches that activate or inhibit GPR34 activity could be used to alter microglial state in neuroinflammatory and neurodegenerative disorders^{16,41,45,69–71}. Whether stimulation or inhibition of GPR34 would be beneficial is likely to depend on the disease pathology, the stage of the disease, and the specific functions of microglia that are being targeted.

Our study is limited to a single disease model and relies primarily on genetic loss-of-function approaches *in vivo*. Although we used lysoPS and Compound 4B to prove GPR34 signaling pharmacologically *in vitro*, future studies should carefully test the impact of pharmacological modulation of GPR34 signaling *in vivo* through both agonist and antagonist approaches. Future research should also address sex-dependent variability and expand to additional disease contexts and examine how GPR34 interacts with other microglial receptors and lipid-sensing

mechanisms^{12,13,32,72–76}. These comparisons will help clarify whether GPR34 acts in parallel or synergistically with pathways mediating microglial homeostasis, lipid metabolism, and the inflammatory response.

Data availability

Code used for this manuscript is available on GitHub (see https://github.com/seanken/GPR34_DAM). Raw sequencing data have been uploaded to the Gene Expression Omnibus (GEO) under accession numbers GSE327711 for the iMGL RNAseq data and GSE327428 for the mouse RNAseq datasets. The processed data will be put in the Broad Single Cell Portal.

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Additional information

Author Contributions

M.S. conceived the project and supervised all aspects of the work. P.K. initiated and oversaw the iMGL project and supervised in vivo data analysis involving *Gpr34* KO and 5xFAD experiments. A.G. designed and conducted iMGL experiments: phagocytosis, RNA-seq, and myelin RNA-seq. M.K. conducted in vivo experiments and contributed to data analysis. Y.Z. co-initiated the iMGL platform, and Y.Z. and Q.X. performed pharmacological validation. W.E.M. generated the *GPR34* knockout clones, co-initiated and co-validated the iMGL platform, performed early iMGL RNA-seq experiment and contributed to in vivo experiments. S.S. analyzed RNA-seq data and contributed to experimental design under the supervision of J.L. J.D., C.D., and N.M. advised and contributed to iMGL experiments under the mentorship of B.S. S.N. performed in vivo immunohistochemistry and analysis under the supervision of H.B. E.Le. initiated in vivo experiments. X.A. conducted the *in vivo* RNA-seq experiments under the supervision of J.L. T.N. and M.D. contributed to iMGL preparation. K.J.S. and D.B. maintained and characterized the *Gpr34* knockout animals. J.Z. contributed to iMGL-associated RNA-seq experiments. A. V. and D.V. performed mass spectrometry proteomics analysis under the supervision of H.K. and S.A.C. E.Lo. and C.B. developed pharmacological compounds under the supervision of D.Mc., S.G., and M.W. D.Mi. contributed to RNA-seq data analysis. M.J. advised on all aspects of projects involving iMGL work. P.S.K., A.G., S.S., Y.L.Z., and M.K. wrote the manuscript with assistance from M.S., and input from all authors

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Additional files

[Supplemental Figure Legends](#) 

[Methods](#) 

[Supplemental Table 1](#) 

[Supplemental Table 2](#) 

[Supplemental Table 3](#) 

[Supplemental Table 4](#) 

[Supplemental Figures](#) 

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Peer reviews

Reviewer #1 (Public review):

Summary:

The authors sought to understand the impact of the decreased expression of the G-protein-coupled receptor GPR34 in Alzheimer's disease (AD). They analyzed the transcriptional impact of GPR34 deficiency in mice and found that it induced a DAM-like phenotype in control mice and enhanced the DAM signature in the AD model 5xFAD, although it did not result in amyloid plaque clearance or gross changes in microglia or astrocytes. Next, the authors developed an in vitro model of GPR34 deficiency using a CRISPR/Cas9 strategy in human iPSCs to introduce functional mutations that resulted in GPR34 protein deficiency in induced microglial cells. In this model, the authors identified myelin as a ligand of GPR34 and showed that GPR34 deficiency resulted in reduced myelin debris engulfment and transcriptional changes related to lysosomal pathways.

Strengths:

The combined strategy of using in vivo and in vitro models of GPR34 depletion is robust, and the transcriptional analyses are thoroughly performed.

Weaknesses:

The paper's two main findings related to the lack of GPR34 (enhancement of DAM signature in vivo and reduced myelin engulfment in vitro) are disconnected. At the very least, the authors should discuss what the relevance of myelin clearance in AD is, but the paper would strongly benefit from a more thorough assessment of the impact of GPR34 deficiency in vivo, particularly because no effects on amyloid clearance were observed. The authors could assess whether GPR34-deficient 5xFAD mice have reduced cognitive performance, which, based on their in vitro findings, could be related to the myelin pathology in AD (previously described: see PMID 36284351). The analysis showing reduced myelin content in GPR34-deficient microglia in vitro is superficial and does not allow for identifying whether GPR34 is related to reduced engulfment or increased degradation, which could be related to the changes in the lysosomal gene CD68 identified in vivo. In addition, it would be interesting to compare the transcriptional profile induced by myelin phagocytosis with that of 5xFAD or AD patients, to

gain insight into the impact of the signature. Finally, the transgenic approach to delete GPR34 *in vivo* could have been complemented with experiments with the GPR34 antagonists (YL-365 or S-E49) or agonist (Compound 4B), possibly helping in identifying the source of discrepancy with previous papers showing that GPR34 promotes amyloid clearance.

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Reviewer #2 (Public review):

Summary:

Using the 5xFAD model in combination with GPR34 mice, the authors explore the function of microglia in the context of neurodegeneration. Using a broad spectrum of methodology, they show that DAM signatures are increased in KO 5xFAD mice. Using several KO clones of GPR34 KO iMGLs and another set of broad methodologies, the authors show that GPR34 is important for microglia homeostasis,

phagocytosis, specifically of myelin. GPR34 KO iMGLs also show a distinct transcriptional response to myelin. Together, they propose that GPR34 limits microglial activation in neurodegeneration.

Strengths:

All methods are state-of-the-art, and the combination of mouse and human microglia responses is a particular strength.

Weaknesses:

No weaknesses were identified by this reviewer.

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Author response:

We greatly thank the Reviewing Editor, Senior Editor and the reviewers for their constructive and thoughtful feedback, as well as for recognizing the significance and strengths of our study. We are very encouraged by overall positive assessment and appreciate these insights to strengthen the manuscript. Below, we outline our plans to address the key points raised in reviewer#1's public review. We also note that reviewer#2 did not identify any weaknesses in the study and are thankful for this positive evaluation.

Point 1: Relating *in vivo* and *in vitro* findings and discussing the relevance of myelin clearance in AD. As suggested, we will elaborate our discussion to cover the relationship between our *in vivo* and *in vitro* findings and present a more unified picture of GPR34 function. We will also highlight the relevance of myelin clearance in Alzheimer's disease independent of amyloid plaque burden.

Point 2: *in vivo* assessments and phenotypes. While we recognize the value of expanding our *in vitro* and cellular findings to *in vivo* and cognitive measures, we believe this additional assessment is beyond the scope of this current study. At least, we will expand our discussion to relate our findings of GPR34-mediated myelin pathology in the contexts of neurodegeneration and cognitive deficiency in AD.

Point 3: Myelin engulfment versus lysosomal degradation. We agree that the clear distinction between impaired myelin engulfment and altered lysosomal degradation is an important point. Although additional experiments to address these scenarios are beyond the scope of the study, we will perform targeted pathway analysis of existing transcriptomic datasets

focusing on the phagosome and lysosomal degradation pathways along with the *in vivo* findings related to CD68, which could offer an interpretation.

Point 4: Comparison of transcriptional signatures. As suggested, we will compare the transcriptional signatures induced by myelin exposure in WT iMGLs with our 5xFAD RNA-seq datasets, as well as publicly available datasets from human Alzheimer's disease microglia, to unravel the potential converged and distinct features.

Point 5: Reconciling our findings with previous GPR34 studies. We will expand our discussion to compare and clarify our current findings with the previous studies and cover potential reasons for those different observations. We will also cover the current limitations of existing GPR34 pharmacological tools compounds, including their limited blood-brain permeability, which precludes the effective use of those tools for proposed *in vivo* studies.

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