

# ***Impact of Early-Life Inflammation and APOE Genotype on the Microglial Omic Profile During Alzheimer's Disease Pathology***

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# 1    **Abstract**

2    Alzheimer's disease (AD) is characterized by amyloid- $\beta$  (A $\beta$ ) plaque deposition, tau pathology, and neuroinflammation, with genetic variation in the apolipoprotein E (APOE) gene representing the strongest risk factor for late-onset AD. Microglia, the brain's resident immune cells, are increasingly recognized as central to disease progression through their roles in neuroinflammation and A $\beta$  clearance. In this study, we investigated how APOE genotype (E3 vs E4) and early-life inflammation interact to shape microglial states in a mouse model of A $\beta$  pathology. Using single-cell RNA sequencing of microglia isolated from EFAD mice treated with lipopolysaccharide (LPS) or vehicle at postnatal day 9, we identified distinct microglial transcriptional states and assessed their proportions and gene expression profiles. E4FAD mice exhibited a higher proportion of disease-associated microglia (DAM) compared to E3FAD mice. Moreover, early-life inflammation induced long-lasting, genotype-specific alterations: LPS reduced the abundance of an NF- $\kappa$ B-enriched Cytokine Response microglial population and downregulated pro-inflammatory genes in E4FAD, but not E3FAD, mice. These findings suggest that early-life immune events interact with APOE genotype to shape microglial responses to amyloid pathology and may offer targets for modulating disease-associated inflammation.

# 15    **Introduction**

## 16    *Alzheimer's Disease and Neuroinflammation*

17    Alzheimer's disease (AD) is a progressive neurodegenerative disorder and the leading cause of dementia worldwide, affecting more than 55 million people as of 2019 (1). It is defined by the accumulation of extracellular amyloid- $\beta$  (A $\beta$ ) plaques and intracellular tau neurofibrillary tangles, which are now considered central pathological hallmarks of the disease (2).

21        In addition to these proteinopathies, AD is characterized by widespread synaptic loss, neuronal degeneration, and a robust neuroinflammatory response. Microglia, the resident immune cells of the central nervous system (CNS), are key contributors to this inflammatory milieu. They participate in A $\beta$  clearance, phagocytosis, synaptic pruning, and cytokine production, and have been implicated in both protective and pathogenic roles.

25       The increasing recognition of neuroinflammation as a core feature of AD has led to renewed interest in  
26       microglial biology, particularly in the context of genetic and environmental risk factors that may shape microglial  
27       responses during disease progression.

28       ***Microglia in Alzheimer's Disease***

29       Microglia are highly dynamic cells that continually survey the brain parenchyma. Their functions are diverse and  
30       include responding to injury, clearing debris, and remodeling synapses. Early frameworks categorized microglia  
31       as either "resting" or "activated," or applied the simplified 'M1/M2' designation. These binary models are now  
32       considered oversimplified and inadequate for describing the true range of microglial phenotypes observed in  
33       health and disease.

34       Advances in single-cell RNA sequencing (scRNA-seq) have transformed our understanding of microglial  
35       heterogeneity. In particular, studies in mouse models of AD have identified a transcriptional program termed Dis-  
36       ease-Associated Microglia (DAM), characterized by downregulation of homeostatic markers such as Tmem119  
37       and P2ry12, and upregulation of genes involved in lipid metabolism, phagocytosis, and inflammation, including  
38       Trem2, Apoe, Cst7, and Tyrobp (3), (4).

39       Subsequent work has revealed additional microglial subsets, including interferon-responsive, antigen-pre-  
40       senting, and proliferative populations. However, there is limited consensus regarding nomenclature or functional  
41       distinctions between these clusters. Moreover, it remains unclear how these states arise, how stable they are  
42       across contexts, and how they relate to disease mechanisms *in vivo*.

43       ***Sex Differences in Alzheimer's Disease***

44       One important but often underappreciated aspect of AD is its differential impact on biological sex. Women  
45       represent nearly two-thirds of AD patients, and several studies have reported that female individuals exhibit  
46       more severe A $\beta$  plaque burden, increased tau pathology, and faster cognitive decline compared to males (5). These  
47       differences may be influenced by sex hormones, immune system regulation, and differential gene expression,  
48       although the underlying mechanisms remain incompletely understood.

49 Sex also interacts with genetic risk factors. The APOE4 allele has been associated with a greater increase in  
50 AD risk in women compared to men (6). In light of these findings, and because immune responses are known to  
51 differ by sex, this study focuses exclusively on female mice. This choice enhances the interpretability of microglial  
52 responses in a biologically relevant context and avoids the confounding influence of sex-dependent variability.

### 53 *APOE Genotype Modulates Microglial Function*

54 The apolipoprotein E (APOE) gene is the strongest known genetic risk factor for late-onset Alzheimer's disease  
55 (AD). The three most common alleles, APOE2, APOE3, and APOE4, differ by two amino acid substitutions. These  
56 differences give rise to distinct protein isoforms that vary in their effects on lipid metabolism and amyloid- $\beta$   
57 ( $\text{A}\beta$ ) clearance (7). Among them, APOE4 is linked to increased  $\text{A}\beta$  aggregation, reduced clearance, disrupted  
58 microglial lipid processing, and altered inflammatory signaling, while APOE2 is considered protective (8), (9).

59 Under normal physiological conditions, APOE is primarily produced by astrocytes. However, in the presence  
60 of  $\text{A}\beta$  pathology, APOE expression in microglia increases substantially. Experimental models using APOE  
61 knockouts or humanized APOE alleles have shown that APOE regulates key microglial functions, including  
62 transcriptional responses to  $\text{A}\beta$ , phagocytic activity, and the production of inflammatory mediators (4), (10).

63 These observations support the hypothesis that APOE isoforms play a critical role in directing microglial  
64 behavior and may influence their progression into DAM or other disease-associated phenotypes.

### 65 *5xFAD and EFAD*

66 Studying the interplay between APOE genotype and microglial activation in humans is difficult due to limited  
67 access to living brain tissue and the confounding effects of postmortem delay. As a result, transgenic mouse  
68 models have become essential tools for investigating AD mechanisms.

69 The 5xFAD mouse model expresses five familial AD mutations in APP and PSEN1, leading to accelerated  
70  $\text{A}\beta$  deposition, gliosis, and synaptic degeneration by six months of age (11). However, this model expresses the  
71 murine Apoe gene, which differs from the human gene in structure and function.

72 To address this limitation, the EFAD model was created by crossing 5xFAD mice with human APOE knock-  
73 in lines. EFAD mice express human APOE2, APOE3, or APOE4 alleles and recapitulate many of the genotype-

74 specific effects observed in humans. E4FAD mice, in particular, exhibit higher plaque load and more pronounced  
75 neuroinflammatory signatures compared to E3FAD or E2FAD mice (12).

76 This model provides a powerful system for dissecting how human APOE isoforms influence microglial  
77 responses to A $\beta$  in vivo.

## 78 *Microglial Priming*

79 In addition to genetic risk, microglial states may be shaped by early-life environmental exposures. One mecha-  
80 nism through which this occurs is innate immune memory, a process by which transient inflammatory stimuli  
81 produce long-lasting changes in microglial function (13).

82 Previous studies have shown that systemic administration of lipopolysaccharide (LPS) during early devel-  
83 opment can lead to persistent alterations in microglial gene expression, inflammatory responses, and behavior  
84 later in life (14). However, these effects are context-dependent and may differ by genetic background, sex, and  
85 timing of exposure.

86 Work from our laboratory has demonstrated that LPS injection at postnatal day 9 (P9) improves long-term  
87 memory performance and enhances microglial internalization of A $\beta$  in 5xFAD mice (15). These findings suggest  
88 that early immune activation may modulate the trajectory of microglial aging and response to pathology, possibly  
89 in a beneficial manner.

## 90 *Study Rationale and Objectives*

91 The current study was designed to test the hypothesis that early-life inflammation interacts with APOE genotype  
92 to shape microglial transcriptional states in the context of A $\beta$  pathology. To investigate this, we used EFAD mice  
93 expressing human APOE3 or APOE4 alleles. At postnatal day 9, animals were treated with LPS or vehicle, and  
94 at six months of age, microglia were isolated for single-cell RNA sequencing.

95 This approach allowed us to examine both cell-type proportions and transcriptional profiles across multiple  
96 experimental conditions. Our aim was to determine whether APOE isoform modifies the microglial response to  
97 early-life priming, with a particular focus on disease-associated and pro-inflammatory transcriptional states.

98 This study is part of a broader investigation integrating transcriptomics, proteomics, histology, and  
99 behavioral analysis to understand how genetic and environmental risk factors converge to influence neuroin-  
100flammation in AD.

## 101 **Materials and methods**

### 102 **Animals**

103 Humanized APOE3 and APOE4 knock-in mice were crossed with the 5xFAD mouse model to generate E3FAD and  
104 E4FAD mice, as previously described (Youmans et al., 2012). Wild-type (WT) littermates were used as controls.  
105 Mice were housed in a controlled environment (12-hour light/dark cycle, constant temperature and humidity)  
106 with free access to food and water. All procedures were conducted in compliance with the European Union  
107 laboratory regulations for animal experiments and were approved by the Animal Research Committee of Lund  
108 University.

**Table 1: Summary of Number of Animals by Genotype and Treatment**

Genotype	Treatment	# of Animals
E3WT	Vehicle	4
	LPS	3
E3FAD	Vehicle	4
	LPS	3
E4WT	Vehicle	4
	LPS	3
E4FAD	Vehicle	4
	LPS	5
Total Animals		30

### 109 **Experimental procedure and sample processing**

110 Only female mice were used in this study, as described in the Introduction. At postnatal day 9 (P9), E3FAD, E4FAD,  
111 and their wild-type (WT) littermates received a single intraperitoneal (i.p.) injection of either lipopolysaccharide  
112 (LPS, 1 mg/kg) or vehicle (0.9% saline) (see Table 1 for group assignments). At six months of age, mice were  
113 euthanized by transcardial perfusion. Brains were rapidly dissected, and the right hemisphere was used for  
114 microglial cell isolation

115 Microglial enrichment was performed using magnetic-activated cell sorting (MACS) with CD11b Mi-  
116 croBeads and LS columns. Enriched microglia were further purified by fluorescence-activated cell sorting (FACS)  
117 using an ARIA III flow cytometer. Cells positive for CD45, CX3CR1, and CD11b were collected for downstream  
118 analyses.

## 119 **Cell Sequencing**

120 From each experimental sample, approximately 2,500 sorted microglial cells were loaded for single-cell capture  
121 and cDNA library preparation using the 10x Genomics Chromium Single Cell 3' v3 reagent kit and workflow.  
122 Individual samples were barcoded using cell-hashing antibodies and pooled into groups of four samples per  
123 sequencing run. Libraries were sequenced on an Illumina platform.

124 Raw sequencing data were processed with CellRanger (10x Genomics, v6.1.2) using the mouse reference  
125 genome GRCm39 (mm39). This pipeline included alignment, filtering, barcode counting, and UMI (unique  
126 molecular identifier) counting to generate feature-barcode matrices for each library.

## 127 **Quality Control, Normalization, and Data Correction**

128 Individual libraries were first filtered to remove cells identified as empty droplets or doublets using the HashSolo  
129 algorithm (16). Additional quality control (QC) filters were applied to each library: cells with fewer than 2,000  
130 total counts, fewer than 300 expressed genes, greater than 5% mitochondrial gene expression, or fewer than 5%  
131 ribosomal gene expression were removed. The 5% mitochondrial threshold was selected based on recommenda-  
132 tions by Osorio and Cai, who showed that this cutoff effectively excludes apoptotic cells in mouse scRNA-seq data  
133 and improves downstream interpretability (17). Genes expressed in fewer than three cells were also excluded. QC  
134 metrics and visualizations for each sample, both pre- and post-filtering, are included in Supplementary Figure 1  
135 and Supplementary Table 1.

136 After filtering, individual libraries were merged into a single dataset using Anndata's concat function with  
137 an outer join along the cell axis and a unique merge for the gene axis. Raw counts were saved as a separate layer  
138 before normalization. Normalization was performed using Scanpy's normalize\_total function followed by log-  
139 transformation with log1p.

140 Following initial normalization, the merged dataset was examined for the presence of contaminating  
141 non-microglial cells. Based on clustering, expression of canonical marker genes, and manual annotation, cells  
142 identified as non-microglia were removed. The dataset was re-normalized after removal of non-microglial cells  
143 using the same normalization strategy.

144 Highly variable genes (HVGs) were then identified using Scanpy's highly\_variable\_genes function with  
145 sample ID as the batch key. These HVGs were used for subsequent dimensionality reduction steps, including  
146 principal component analysis (PCA).

147 The merged dataset was assessed for batch effects using PCA. Several batch correction methods were tested,  
148 including Harmony (integration via latent space correction), BBKNN (graph-based nearest neighbor correction),  
149 and ComBat (empirical Bayes adjustment). Ultimately, batch effects were not observed to be significant, and no  
150 batch correction was applied.

151 A high contribution of mitochondrial genes to variance across cells was observed during HVG and PCA  
152 analysis. To reduce this effect, mitochondrial gene expression was regressed out using Scanpy's regress\_out  
153 function prior to downstream analyses.

**Table 2: Summary of Datasets Before and After Quality Control**

Library	Before		After	
	# of Cells	# of Genes	# of Cells	# of Genes
D1	3403	33989	2903	14987
D2	5041	33989	4698	15514
D3	5029	33989	4081	15463
D4	3892	33989	3558	15052
D5	3825	33989	3606	15260
D6	4408	33989	3603	15788
D7	3643	33989	3103	14935
D8	3903	33989	3601	15926
Merged	-	-	29153	17341

## 154 Clustering and Annotation

155 Principal component analysis (PCA) was computed on normalized, log-transformed data. Neighborhood graphs  
156 were constructed using the top 25 principal components as input after analyzing the scree plot, and uniform

157 manifold approximation and projection (UMAP) embeddings were calculated with a min\_dist parameter of 0.5  
158 to preserve both local and global structures.

159 Leiden clustering (18) was performed iteratively across a range of resolutions (0.1–1.9 in steps of 0.1). UMAPs  
160 colored by cluster identities were visually inspected at each resolution to identify regions of high heterogeneity  
161 and monitor for under- and overclustering. Annotation started with the highest resolution clustering to capture  
162 fine-grained heterogeneity and then merged clusters that showed high similarity in their gene expression  
163 profiles. Overclustering was identified when newly created clusters did not significantly differ from neighboring  
164 clusters in their top differentially expressed genes (DEGs).

165 DEGs were identified using the Wilcoxon rank-sum test (rank\_genes\_groups function). DEGs were calcu-  
166 lated for each cluster against the total dataset and neighbouring clusters to merge clusters with insufficiently  
167 distinct expression profiles. Expression profile annotations were assigned based on the combination of differen-  
168 tial expression profiles, cluster relationships, and comparison to canonical gene signatures described in previous  
169 studies, including Keren-Shaul et al. (3), Olah et al. (19), Prater et al. (20), Mancuso et al. (21), Sala-Frigerio et  
170 al. (22), Mathys et al. (23), Millet et al. (24), (25), (26).

171 Clusters were annotated into 9 expression profiles; Homeostatic, DAM, MHC-II/Antigen Presentation DAM,  
172 Cytokine Response, Cycling(G2M/Cdk1+), BAM-like, Neuronal surveillance/Ribosomal biogenesis, DAM with  
173 Ribosomal upregulation, and Immediate Early Gene (IEG) Enriched Homeostatic. Naming of profiles was based  
174 on similarity to previously described profiles in the literature, though should not be considered a formal defin-  
175 ition.

## 176 Cluster Proportions

177 The proportion of each cluster was calculated as the number of cells in the cluster divided by the total number  
178 of cells in the sample. A three-way ANOVA was performed to assess the effect of genotype (E3 vs E4), treatment  
179 (LPS vs Vehicle), and disease status (FAD vs WT) on the proportion of each cluster. An ordinary least squares  
180 (OLS) linear regression model was fit to the data with the proportion of each cluster as the dependent variable  
181 and the variable main effects and interactions as predictors. Tukey's Honest Significant Difference (HSD) test

182 was used for post-hoc pairwise comparisons between conditions. All statistical analyses were performed using  
183 the statsmodels package in Python.

## 184 **Pseudobulk Analysis**

185 Pseudobulk analysis was performed to more robustly identify DEGs between condition groups as it has been  
186 shown to significantly reduce the number of false positives and increase the accuracy of subsequent enrichment  
187 analyses as compared to the Wilcoxon rank-sum test (27). The Python package ‘Decoupler’ was used to generate  
188 pseudobulk profiles for each individual mouse using the ‘sum’ method. The pseudobulk profiles were then used to  
189 identify DEGs between groups using ‘PyDeseq2’, a python implementation of the DESeq2 algorithm (28,29). The  
190 Wald test was used to generate p-values with cook’s filtering and correction for multiple testing using Benjamini-  
191 Hochberg method. The model was fit with disease status (FAD vs WT), APOE genotype (E3 vs E4), and treatment  
192 (LPS vs Vehicle) as factors with their interactions, and specific comparisons were extracted using the ‘results’  
193 function. Genes were considered differentially expressed if they had an adjusted p-value < 0.05 and a log2 fold  
194 change > |0.25|. The pseudobulk DEGs were then used for downstream analysis including Gene Ontology (GO)  
195 enrichment analysis.

## 196 **Gene Set Enrichment Analysis (GSEA)**

197 Gene Set Enrichment Analysis (GSEA) is a statistical method used to determine whether selected sets of genes  
198 show statistically significant differences between two biological states (30). Rather than focusing only on statis-  
199 tically significant DEGs, as other methods do, GSEA evaluates the entire set of genes ranked by a metric which  
200 reflects their different expression between conditions, thereby better capturing the pathway-level enrichment  
201 differences.

202 For each contrast of interest, genes were ranked by their Wald test statistic derived from the pseudobulk  
203 differential expression analysis. GSEA was then performed using the implementation provided in the Decoupler  
204 package (28), with the MSigDB Hallmark gene sets (31) as the reference collection. This approach enabled the  
205 identification of pathways significantly enriched in up- or down-regulated genes across our conditional contrasts  
206 of interest.

207 **Proteomic Analysis**

208 *Sample Preparation*

209 50 µg of protein was digested on S-Trap micro spin columns (ProtiFi) with sequencing-grade trypsin (1:25)  
210 overnight at 37 °C following the manufacturer's protocol.

211 *LC–MS/MS Acquisition*

212 Peptides (500 ng) were analyzed on an Evosep One–timsTOF HT system in diaPASEF mode (16 windows, 400–  
213 1 200 m/z, 0.60–1.60 1/K<sub>0</sub>; 1.8 s cycle), using 0.1 % formic acid mobile phases.

214 *Data Quantification*

215 Raw files were processed in Spectronaut v19 (directDIA) using default MS2 quantification and MaxLFQ. The  
216 UniProt mouse reference proteome (17 184 entries), supplemented with human APOE3, APOE4, and 5×FAD APP/  
217 PSEN1 sequences, served as the search database. Carbamidomethylation of cysteine and oxidation of methionine  
218 were set as fixed and variable modifications, respectively; up to two missed cleavages were allowed. False  
219 discovery rate was controlled at 1 % for both peptide and protein levels.

220 (Full protocol provided in Supplementary Methods)

221 *Data Analysis*

222 Log<sub>2</sub>-transformed normalized label-free quantification (LFQ) values from Spectronaut v19 were imported into  
223 R (v4.x), reshaped and annotated with sample metadata using tidyverse and dplyr [(32)]. Metadata fields included  
224 sample ID, genotype, treatment, age, sex and batch. A SummarizedExperiment object was generated with DEP  
225 (v2.0.0) [(33)] for downstream analyses.

226 Comprehensive quality control was conducted within DEP. Density and box plots of log<sub>2</sub> intensities were  
227 inspected to confirm consistent normalization across samples. Protein detection frequency was assessed by  
228 counting proteins detected in n samples, and the distribution was visualized to evaluate assay coverage. Missing  
229 data patterns were explored by generating heatmaps of missingness and plotting the fraction of missing values  
230 against intensity. On the basis of these diagnostics, missing values were classified as Missing Not At Random  
231 (MNAR) and found to be enriched at low intensities. Imputation was performed using the MinProb algorithm (q

232 = 0.5) as implemented in DEP. Additional exploratory analyses included Principal Component Analysis (PCA),  
233 hierarchical clustering, heatmaps of Pearson correlation coefficients between samples.

234 Differential abundance analysis was conducted with limma [(34)]. Linear models were fitted to  $\log_2$  inten-  
235 sities with genotype, treatment and their interaction specified as factors. An empirical Bayes procedure with  
236 mean-variance trend fitting and robust hyperparameter shrinkage was applied. Contrasts were defined to test  
237 the effect of LPS within APOE3, the effect of LPS within APOE4, and the interaction between genotype and  
238 treatment. Proteins with nominal  $P < 0.05$  were selected for visualization. Volcano plots were generated using  
239 ggplot2, displaying  $-\log_{10}(P)$  versus  $\log_2$  fold change with top hits annotated. Concordance with transcriptomic  
240 data was assessed by plotting proteomic  $\log_2$  fold changes against matching RNA-seq contrasts and calculating  
241 Spearman correlation coefficients.

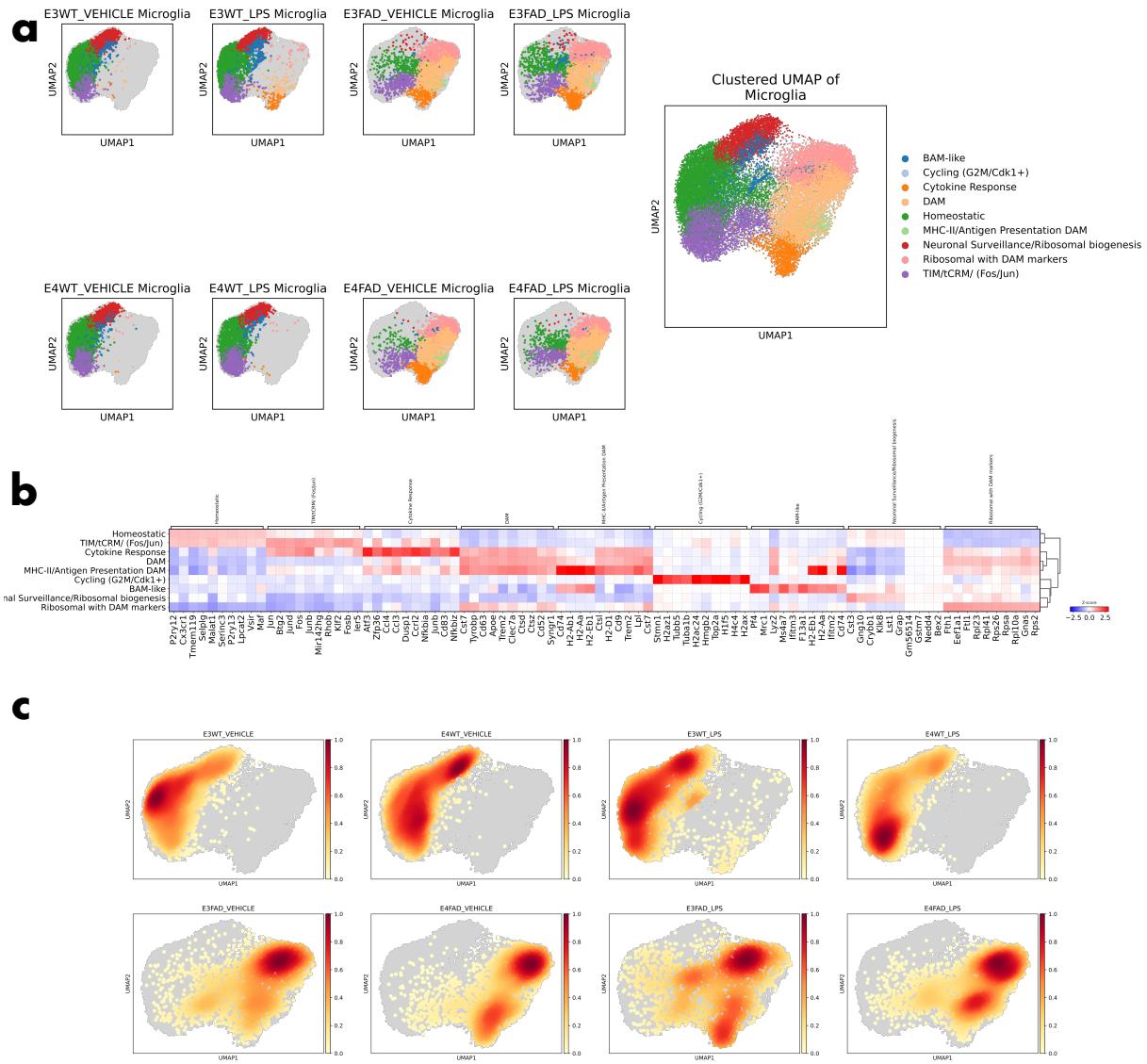
242 Pathway enrichment analysis was performed using Camera [(35)] in limma against KEGG pathways from  
243 the Molecular Signatures Database (MSigDB) C2:CP (curated canonical pathways) [(31)]. Camera's competitive  
244 gene set test, which estimates inter-gene correlation from the dataset to control type I error, was executed with  
245 default settings using the full vector of  $\log_2$  fold changes as input. Pathways were ranked by false discovery rate  
246 for interpretation.

## 247 **Results**

### 248 *Cluster Expression Profiles*

249 Following dimensionality reduction and Leiden clustering, we identified nine distinct microglial expression  
250 profiles based on differentially expressed genes (DEGs) between clusters and reference to canonical expression  
251 signatures described in previous studies. Cluster annotation was guided by comparisons to known microglial  
252 states in both mouse and human brain studies, including those by Keren-Shaul et al. (3), Sala-Frigerio et al.  
253 (22), Olah et al. (19), Mathys et al. (23), and others. The final annotated expression profiles were: Homeostatic,  
254 DAM, MHC-II/Antigen Presentation DAM, Cytokine Response, Cycling (G2M/Cdk1+), BAM-like, Neuronal  
255 Surveillance/Ribosomal Biogenesis, Ribosomal DAM, and Immediate Early Gene (IEG) Enriched Homeostatic.

These expression profiles are visualized in Figure 1. The UMAP plots (Figure 1a) show cluster organization across the entire dataset and by condition. Figure 1b displays the top 10 DEGs for each cluster when compared to the rest, and Figure 1c shows density plots of cluster distributions per condition.



**Figure 1: Distinct Expression Clusters and Their Proportions by Condition**

**a.** UMAP visualizations of the dataset colored by manually annotated expression profiles, entire dataset, and by condition. **b.** Heatmap of the top 10 differentially expressed genes for each cluster expression profile showing scaled log-normalized expression values (z-scores) **c.** UMAP density plots for each condition.

259 The Homeostatic cluster was characterized by high expression of canonical microglial maintenance genes,  
260 including P2ry12, Tmem119, and Cx3cr1, and served as a reference for identifying more activated or altered  
261 states. The IEG cluster shared elevated expression of homeostatic markers but also showed upregulation of

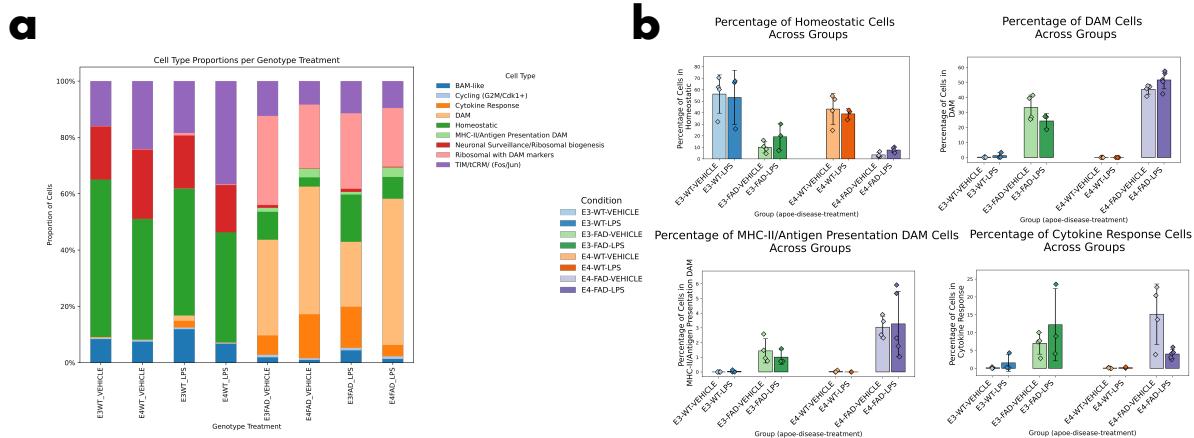
262 immediate early genes such as Fos, Jun, and Egr1, a pattern that has been interpreted as both a stimulus-  
263 responsive microglial state and, alternatively, as a dissociation-induced artifact (24,36).

264 Two small clusters were also identified. The Cycling cluster was defined by elevated expression of cell  
265 cycle and proliferation-associated genes such as Stmn1, Top2a, and H2az1, consistent with previously reported  
266 populations of proliferative microglia in AD models and aged brain tissue (22,26,37). The BAM-like (Border-  
267 Associated Macrophage-like) cluster was characterized by high expression of perivascular macrophage markers  
268 including Pf4, Mrc1, and Ms4a7. Although microglial enrichment was performed during FACS, this population  
269 likely reflects a small number of CNS-associated macrophages retained during sorting.

270 The DAM (Disease-Associated Microglia) cluster showed a transcriptional profile closely matching that  
271 originally described by Keren-Shaul et al. (3), including high expression of Trem2, Apoe, Cst7, Tyrobp, and Clec7a,  
272 accompanied by suppression of homeostatic genes. Three additional clusters were identified with overlapping  
273 DAM features but distinct gene expression signatures. The Cytokine Response cluster exhibited elevated expres-  
274 sion of inflammatory chemokines and NF- $\kappa$ B target genes such as Ccl3, Ccl4, and Il1b. The MHC-II/Antigen  
275 Presentation DAM cluster was enriched for antigen presentation genes including Cd74, H2-Ab1, H2-Aa, and H2-  
276 Eb1. The Ribosomal DAM cluster retained DAM-like features with additional upregulation of ribosomal genes.

277 ***Expression Profile Proportions Across Conditions***

278 After identifying different expression profiles in the dataset, the relative abundance of each cell type was  
279 assessed across experimental conditions. The proportions observed in the eight experimental groups are shown  
280 in Figure 2a, with absolute cell counts presented in Supplementary Figure 2 and corresponding values listed in  
281 Supplementary Table 2. Disease versus wild-type is the main cause of variation in cell type composition, with  
282 the most pronounced differences observed in the homeostatic and DAM clusters. Homeostatic cells accounted  
283 for 39–56% of the population in WT mice, compared to only 3–17% in FAD mice, representing a statistically  
284 significant reduction (ANOVA  $p < 1e-8$ ; linear model coefficient = +46.2%,  $p < 0.001$ ). The APOE genotype was  
285 not associated with a statistically significant change in homeostatic cell proportions, nor was there a significant  
286 difference with LPS treatment.



**Figure 2: Changes in Cluster Proportions by Condition**

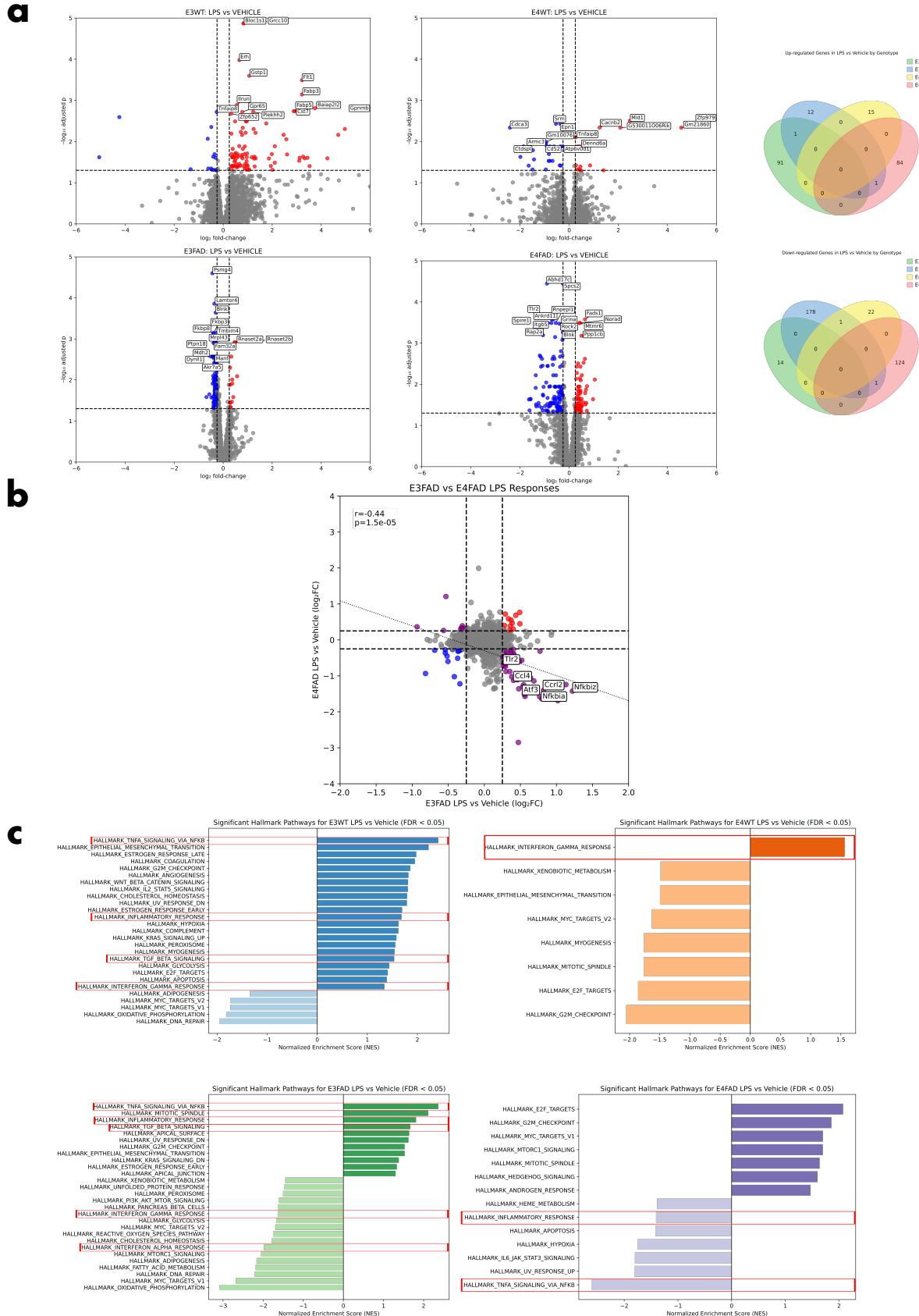
a. Stacked Bar plot of the proportion of each cluster in each condition. b. Bar plots of the proportion of specific clusters in each condition, significant differences determine by three way anova and Tukey's post-hoc test and indicated by asterisks.

287 The DAM cluster was absent in all WT groups except for a small proportion (1.9%) in the E3WT LPS group  
 288 and ranged from 23-52% in FAD mice. Additionally, E4FAD mice have a larger proportion of DAM microglia  
 289 than E3FAD mice (interaction coefficient = +12.3%, p = 0.012), which is consistent with previous single cell  
 290 studies. The three clusters representing more “activated” states similar to DAM (Cytokine Response, MHC-II/  
 291 Antigen Presentation DAM, and Ribosomal DAM), also show zero or very low proportions in WT mice, and are  
 292 significantly increased in FAD mice.

293 The Cytokine Response cluster shows an interesting pattern in which the E4FAD LPS group has a signifi-  
 294 cantly lower proportion of cells compared to the E4FAD vehicle group (Tukey p-adj = 0.028), while the E3FAD  
 295 LPS group is not significantly different from the E3FAD vehicle group (Tukey p-adj = 0.807). This suggests an  
 296 APOE isoform-specific effect of LPS priming on this microglial population. This interpretation is supported by  
 297 a statistically significant three-way interaction term in the ANOVA (p = 0.038) and a notable effect size in the  
 298 linear model (coefficient = -15.1%, p = 0.038), indicating that LPS treatment reduces the proportion of Cytokine  
 299 Response cells specifically in E4FAD mice.

300 ***E4FAD but Not E3FAD or WT Mice Exhibit Reduced NfkB-related Expression after LPS Priming***

302 To more robustly identify differentially expressed genes (DEGs) between conditions, pseudobulk analysis was  
303 performed on the dataset. This involves summing the expression each gene in all cells from an individual mouse  
304 to create a single expression profile, aiding in overcoming the sampling variability in single cell RNA sequencing.  
305 The pseudobulk profiles can then be used to identify DEGs with methods typically used for bulk RNA sequencing  
306 data, such as DESeq2 which produces fewer false positives than the more common Wilcoxon rank-sum test  
307 used in single cell analysis. DEGs were identified between the LPS and vehicle treatment groups in the four  
308 genetic backgrounds, the results of which are visible in the volcano plots in Figure 3a. There are no more than 1  
309 significant DEG which is shared between any of the comparisons. However, as a general pattern it can be seen  
310 that the FAD mice have a much larger number of downregulated genes in the LPS treatment group compared to  
311 the vehicle group, while the WT mice show a much smaller number of downregulated genes.



**Figure 3: Differentially expressed Genes and Pathways in LPS versus Vehicle Treated Mice**

a. Volcano plots of pseudobulk DEGs in LPS versus vehicle comparisons in the four genotypes with top genes labelled. b. Correlation plot of LogFC values of genes in LPS versus vehicle treatment in E3FAD compared to E4FAD. Pearson's correlation computed on genes above the logFC threshold of 0.25 c. GSEA of significant pathways in the LPS versus vehicle comparisons computed on Wald test statistics.

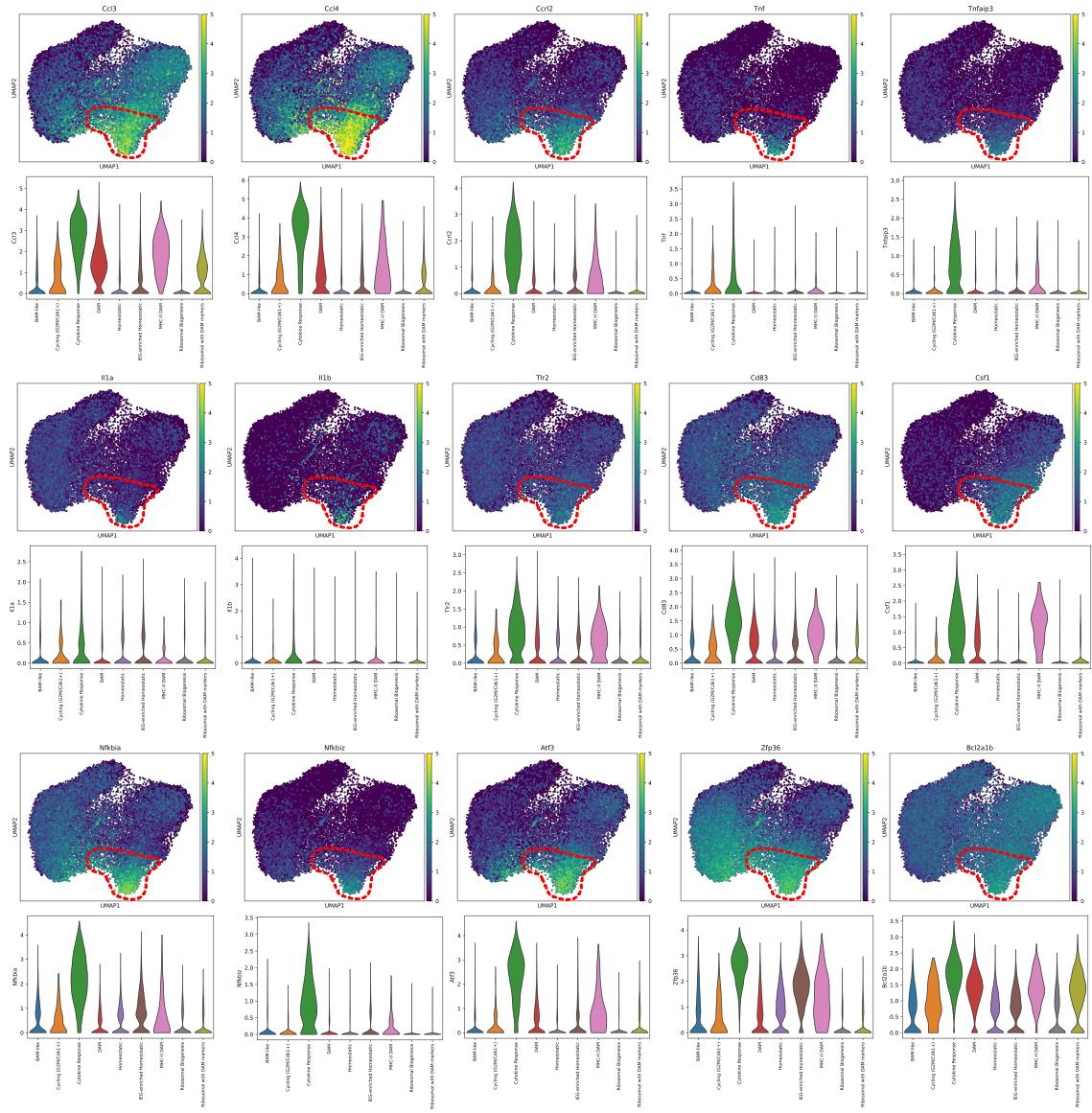
312 When the E3FAD and E4FAD LPS treatment group DEGs are compared, a statistically significant correlation is  
313 observed (Pearson's  $r = -0.44$ ,  $p = 1.5\text{e-}5$ ), indicating that genes that are upregulated in E3FAD mice are likely  
314 to be downregulated in E4FAD mice. A number of these genes are those associated with the NfkB pathway,  
315 including Ccl4, Tlr2, and Nfkbia. Based on the pseudobulk DEGs, Gene Set Enrichment Analysis (GSEA) was  
316 performed to identify pathways that were significantly enriched in the LPS treatment groups. The results (Fig 3c)  
317 show that the top upregulated pathways in both E3WT and E3FAD groups are TNF-a signaling via NfkB, while  
318 it is the top downregulated pathway in the E4FAD group. It does not appear in the significant pathways in the  
319 E4WT group, however the top upregulated pathway in this group is Interferon gamma response. Additionally,  
320 the E3WT and E3FAD groups show significant upregulation of the Inflammatory response pathway and TGF  
321 beta signaling, while the E4FAD group shows downregulation of the Inflammatory response pathway and no  
322 upregulation of inflammatory pathways.

### 323 *Cytokine Response Microglia*

324 Given that the dampening of NF- $\kappa$ B-related pathways in the E4FAD LPS group coincided with a reduced  
325 proportion of Cytokine Response microglia, the transcriptional profile of this cluster was examined to assess its  
326 potential contribution to the observed pseudobulk differences.

327 The Cytokine Response cluster was marked by elevated expression of genes associated with pro-inflam-  
328 matory signaling and canonical NF- $\kappa$ B pathway activation. These included the chemokines Ccl3 and Ccl4, the  
329 interleukins Il1a and Il1b, and the TNF family member Tnf. Several key regulators and targets of NF- $\kappa$ B signaling,  
330 including Nfkbia, Nfkbiz, and Tnfaip3, were also upregulated. Additional genes enriched in this cluster were  
331 involved in stress response (Atf3, Gadd45b), immune regulation (Cd83, Zfp36, Bcl2a1b), pattern recognition  
332 (Tlr2), cytokine signaling (Csf1), and chemokine receptor activity (Ccrl2).

333 These expression patterns are shown in Figure 4. UMAP projections reveal that these transcripts are highly  
334 expressed within the Cytokine Response cluster, with limited expression in other microglial states. Violin plots  
335 further illustrate the cluster-specific enrichment of these genes.



**Figure 4: Genes in the Cytokine Response Cluster**

UMAP plots of the dataset colored by the expression of specific genes which are differentially upregulated in the Cytokine Response cluster and which are part of the NF- $\kappa$ B pathway paired with the violin plots in all clusters.

336 The selective expression of inflammatory genes within this cluster, combined with its reduced abundance in  
 337 E4FAD mice following LPS treatment, suggests that changes in this microglial state contribute to the largest  
 338 observed pathway-level differences in the more conservative pseudobulk analysis.

339 In addition to the upregulation of pro-inflammatory signaling genes, the Cytokine Response cluster was  
 340 found to show elevated expression of several immediate early genes (IEGs), including Fos, Jun, Egr1, and  
 341 Dusp1. This overlap prompted further comparison with the IEG-enriched cluster, which also showed increased

342 expression of these same IEGs relative to the rest of the dataset. Despite this similarity, clear differences were  
343 observed between the two clusters. The IEG-enriched cluster retained high expression of canonical homeostatic  
344 markers such as Tmem119, Cx3cr1, and P2ry12, which were largely absent in the Cytokine Response cluster. In  
345 contrast, the Cytokine Response cluster was enriched for DAM-associated genes including Apoe, Clec7a, Trem2,  
346 and Ctsd, both in comparison to the total population and when directly compared to the IEG-enriched cluster.

When compared to the DAM cluster, the Cytokine Response cluster remained distinct. The DAM cluster did not show enrichment for IEGs or NF- $\kappa$ B pathway-associated transcripts, suggesting that the Cytokine Response cluster represents a distinct activation state with overlapping features of both DAM and IEG-enriched microglia. These relationships are visualized in the dot plot in figure 5.

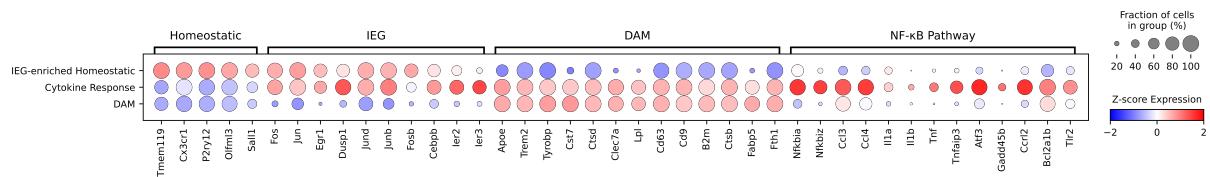


Figure 5: Comparison of Overlapping and Distinct Features Between Microglial States

Dot plot showing the expression of immediate early genes (IEGs), homeostatic markers, DAM-associated genes, and NF- $\kappa$ B pathway-related transcripts across the Cytokine Response, IEG-enriched, and DAM clusters. Dot size represents the fraction of cells expressing each gene, calculated using a threshold of log-normalized expression > 0 (equivalent to raw counts  $\geq 1$ ). Color indicates average expression level scaled across clusters (Z-score normalized).

351 *Proteomic Analysis*

352 The relationship between mRNA abundance and protein levels in vivo is complex and influenced by translational  
353 regulation, post-translational modifications, and protein degradation. Therefore, transcript levels often do not  
354 directly predict protein abundance, with reported correlations varying widely across tissues and cell types and  
355 frequently being low (Liu et al. 2016). To corroborate and extend our transcriptomic findings, mass spectrometry-  
356 based proteomic analysis was performed on the same individuals used for single-cell sequencing.

357 A total of 8 065 proteins were quantified across all samples. UniProt mouse identifiers were mapped to  
358 gene symbols (GRCm39) using the R package org.Mm.eg.db, allowing direct comparison with the transcriptomic  
359 dataset. The proteomic cohort comprised the female whole-cortex samples from LPS and vehicle groups of E3FAD  
360 and E4FAD mice ( $n = 5$  per condition), plus additional samples not included in the transcriptomics (yielding

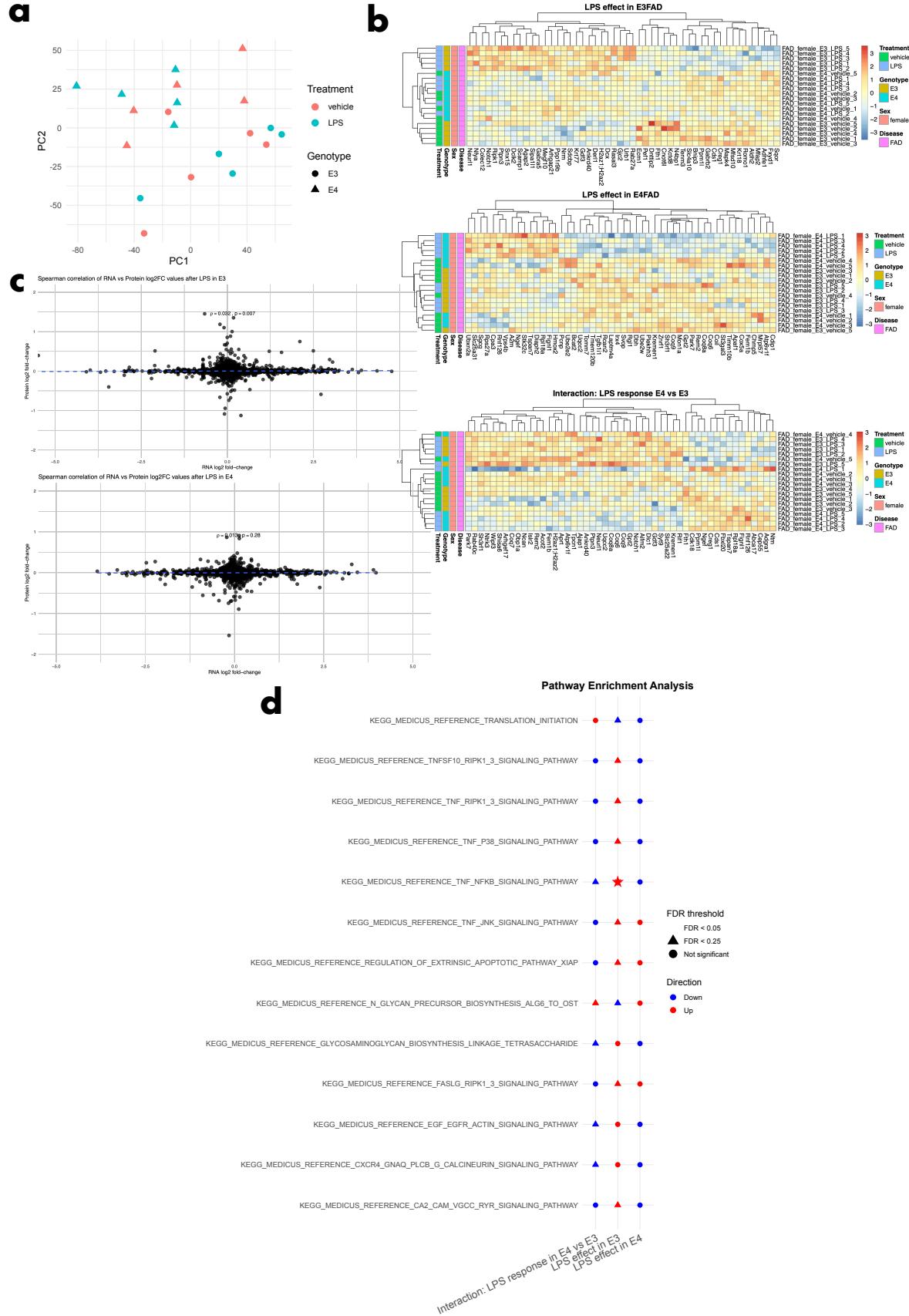
361 up to five replicates per group). Unlike the microglia-enriched preparations used for transcriptomics, proteomic  
362 profiling was performed on whole cortex. Although wild-type and male cohorts were also analyzed, only E3FAD  
363 and E4FAD mice are included here to align with the transcriptomic analysis.

364 Principal component analysis (PCA) of the log<sub>2</sub>-transformed LFQ intensities revealed clear separation by  
365 APOE genotype but no discernible clustering by treatment (Fig 6a), indicating that LPS priming did not induce  
366 a major shift in the cortical proteome. Consistent with this, differential abundance analysis using limma on  
367 imputed LFQ values identified no proteins with significant changes (FDR < 0.05) between LPS and vehicle in  
368 either genotype.

369 To assess concordance between transcriptomic and proteomic responses to LPS, we compared the log<sub>2</sub> fold  
370 changes of differentially expressed genes (DEGs) from the RNA-seq analysis to the corresponding values for their  
371 protein products (n = 7 051 matched identifiers). Spearman correlation coefficients were  $\rho = 0.032$  ( $p = 0.007$ ) for  
372 E3FAD and  $\rho = 0.013$  ( $p = 0.028$ ) for E4FAD (Fig 6c), indicating extremely weak correlation between mRNA and  
373 protein fold-change directions.

374 Despite the lack of individually significant proteins and the low global correlation, differentially abundant  
375 proteins can be visualized in a clustered heatmap (Fig 6b). To determine whether pathway-level signals might  
376 emerge, we applied Camera to test KEGG Medicus Reference pathways for enrichment against the full set of log<sub>2</sub>  
377 fold changes. This approach can detect changes in pathway enrichment even when individual proteins fail to  
378 reach statistical significance by leveraging coordinated shifts across gene sets.

379 After multiple-testing correction, the “TNF NFKB signaling pathway” was the only KEGG pathway signif-  
380 icantly enriched (FDR < 0.05) in the upregulated direction for E3FAD mice treated with LPS (Fig 6d). This  
381 finding aligns with our transcriptomic GSEA results, which also highlighted TNF-NF-κB activation in E3FAD LPS  
382 samples. In the E4FAD LPS group, the pathway showed a trend toward downregulation but did not reach FDR  
383 < 0.05. The interaction contrast, however, met FDR < 0.25 for downregulation, pointing to genotype-dependent  
384 changes in NF-κB signaling after LPS treatment.



**Figure 6: Proteomic Analysis of LPS effect in E3/E4FAD**

**a.** PCA plot of proteomic data for E3FAD and E4FAD female mice in LPS and Vehicle treatment groups. **b.** Heatmap of Z-score scaled logFC of proteins with smallest nominal P values for the LPS treatment effect in both genotypes, and the interaction term which indicates those which

have the biggest different treatment effect between the two genotypes. Rows correspond to individual samples. both Rows and Columns are ordered by heirarchical clustering. **c.** Scatter plots comparing Logfc values after LPS treatment in both genotypes in the proteomic data against the transcriptomic data. Only proteins/genes which could be matched between both were kept (n = 7051). Spearman correlation calculated (E3FAD: $\rho=0.032$ , p = 0.007; E4FAD: $\rho=0.013$ , p=0.028) **d.** Dot plot of all pathways which have a FDR < 0.25 in atleast one contrast. Dot coloured by direction of enrichment and dot shape corresponds to FDR values.

## 385 Discussion

### 386 *Microglial Heterogeneity in Alzheimer's Disease*

387 Neuroinflammation has become recognized as a central phenomenon in the pathogenesis of Alzheimer's disease  
388 (AD), both regulating and responding to amyloid- $\beta$  production, plaque formation, tau hyperphosphorylation, and  
389 synaptic and neuronal loss, within a complex and dynamic system that remains incompletely understood (38).  
390 While all central nervous system (CNS) cell types have been implicated, accumulating evidence has positioned  
391 microglia—the resident innate immune cells of the brain—as central players in these processes. As research into  
392 microglial involvement in AD has expanded, so too has the understanding of their diverse functional states.  
393 However, the molecular mechanisms governing transitions between these states remain poorly defined.

394 Advances in laboratory techniques and in particular single-cell transcriptomics have given us an entirely  
395 new view of microglial heterogeneity. The outdated binary classification of microglia into “resting” or “activated”  
396 states has been replaced by an understanding of multiple distinct transcriptional states influenced by disease  
397 progression, brain region, age, microenvironment, and genetic background. This shift has been driven by  
398 studies describing specific microglial activation profiles, among which the Disease-Associated Microglia (DAM)  
399 program, described by Keren-Shaul et al. (3), remains the most influential. This transcriptional program was  
400 proposed to occur in two stages, dependent on TREM2 signaling, and in response to amyloid deposition.  
401 Subsequent studies have identified a broader array of microglial states, including cycling, antigen-presenting,  
402 phagocytic, and various pro-inflammatory states. The current challenge in this area of research is integrating  
403 a rapidly expanding body of evidence produced with varied model systems, methodologies, nomenclatures and  
404 sometimes conflicting interpretations.

405 APOE genotype remains the strongest genetic risk factor for late-onset Alzheimer's disease, and in the  
406 context of microglial heterogeneity, its importance has become increasingly apparent. APOE is highly expressed

407 by microglia in response to amyloid pathology and has been shown to modulate a number of microglial func-  
408 tions including phagocytosis, lipid metabolism, and inflammatory signaling. The data presented here provide  
409 additional evidence for APOE-dependent modulation of microglial state. As expected, homeostatic microglia  
410 represented the majority population in wild-type mice and were nearly absent in the presence of amyloid  
411 pathology. FAD mice showed a marked shift toward DAM and related activated states, with a significantly higher  
412 proportion of DAM microglia observed in E4FAD compared to E3FAD mice. This finding is consistent with  
413 previous studies using humanized APOE models.

#### 414 *Early-Life Immune Priming Alters Microglia Trajectory*

415 LPS priming of microglia has been shown to influence their function both acutely and over extended periods  
416 following the initial insult (39). Unpublished data from members of our lab have demonstrated that LPS adminis-  
417 tration at postnatal day 9 alters the microglial response to amyloid pathology later in life. Changes were observed  
418 in the transcriptional levels of specific cytokines, and a microglial population not present in vehicle-treated mice  
419 was identified ((15), preprint). These findings, combined with the well-established role of APOE in modulating  
420 microglial state and inflammatory signaling, motivated the present analysis to investigate how human APOE  
421 isoforms may interact with early-life immune priming to shape microglial phenotypes in the context of AD-  
422 related pathology.

423 The data presented here provide additional evidence for the impact of APOE genotype on microglial pheno-  
424 types and the modulatory effect of LPS priming. As expected, a majority of microglia in non-disease (WT) mice  
425 were assigned to the homeostatic cluster, with near-zero representation in the disease-associated transcriptomic  
426 profiles. In contrast, FAD mice exhibited a substantial shift, with large proportions of their microglial populations  
427 classified into disease-associated clusters and a corresponding reduction in homeostatic cells. An increased  
428 proportion of microglia in the DAM cluster was observed in E4FAD mice compared to their E3FAD counterparts,  
429 consistent with previous studies using mice expressing human APOE alleles.

#### 430 *Genotype-Specific Modulation of Inflammatory Signal*

431 The analysis shows a genotype-specific priming effect on the expression of particular cytokines and inflamma-  
432 tory genes, primarily those in the canonical NF-κB signaling pathway. In mice treated with LPS, a downregulation

433 of these genes was observed specifically in E4FAD animals, while an upregulation was seen in the LPS-treated  
434 E3FAD group (Fig. 3). The genes associated with these pathways were not expressed uniformly across the  
435 microglial population, indicating that the observed transcriptional changes were not global but instead reflect the  
436 behavior of a specific microglial subset. This subset, identified as the Cytokine Response cluster, was not present  
437 in non-disease mice, indicating that its emergence is dependent on amyloid pathology. Cluster-level analysis  
438 suggests that the observed pathway-level effect in E4FAD mice treated with LPS is driven by a reduction in the  
439 proportion of microglia occupying this state.

440 To determine whether these APOE- and treatment-specific transcriptional signatures translate to protein  
441 abundance, we performed LC–MS/MS–based proteomics on the matching cortex samples. PCA of LFQ intensities  
442 separated samples by APOE genotype but not by LPS treatment, and limma analysis revealed no individual  
443 proteins with FDR < 0.05. Nonetheless, pathway enrichment on the fold-change values identified the TNF-NF-  
444 kB signalling cascade as significantly upregulated in E3FAD + LPS (FDR < 0.05) and trending down in E4FAD +  
445 LPS (interaction FDR < 0.25), mirroring the observed transcriptomic pattern between genotypes.

#### 446 *Contextualizing the Cytokine Response Cluster*

447 A Cytokine Response cluster, defined by increased expression of cytokines, chemokines, and NF- $\kappa$ B-related  
448 genes, has been observed in other studies. Lee et al. (10) used E3FAD and E4FAD mice challenged with LPS and  
449 performed single-cell sequencing 24 hours later; a cluster enriched for NF- $\kappa$ B–related transcripts was identified,  
450 with higher abundance in E3 compared to E4. Mancuso et al. (21) identified a transcriptionally similar population  
451 in their xenograft model, significantly enriched in the presence of amyloid pathology, although no APOE  
452 genotype–specific differences were reported. Hammond et al. (25) analyzed healthy mice across a range of ages  
453 and identified microglial clusters with similar chemokine and cytokine expression profiles, which increased in  
454 abundance with age. This microglial phenotype has now been identified across multiple models and conditions;  
455 however, its functional role in Alzheimer’s disease remains unclear.

456 Beyond the identification of this cluster, cytokine signaling more broadly remains poorly characterized  
457 in the context of AD. Cytokines have been proposed to contribute to increased amyloid- $\beta$  production and  
458 accumulation (40), and microglial phagocytosis of amyloid- $\beta$  has been shown to induce cytokine release (41).

459 Other studies have reported that interleukin-1 $\beta$  reduces microglial clearance of amyloid- $\beta$  (42) and that cytokine  
460 production is associated with neuronal loss. However, there is also evidence that LPS-induced inflammation can  
461 promote amyloid- $\beta$  clearance (43). These findings highlight the complexity of cytokine signaling in AD, which  
462 is governed by context-specific feedback loops that depend critically on the specific cytokines, chemokines, and  
463 signaling pathways involved, as well as their timing and intensity

464 Continued investigation of these microglial populations will be important for understanding their function,  
465 how they develop and progress, and how they transition across brain regions and in response to disease states.  
466 The analysis presented here suggests that this population can be modulated by pre-symptomatic intervention,  
467 which may represent a viable therapeutic strategy. This transcriptomic analysis forms part of a larger study  
468 examining the interaction between early-life LPS priming and APOE genotype. Unpublished data from this study  
469 indicate improved short-term memory performance in LPS-treated E4FAD mice, an effect not observed in their E3  
470 counterparts. Taken together, these findings suggest that modulating microglial phenotype at an early timepoint  
471 may yield beneficial effects on Alzheimer's disease-related outcomes.

## 472 *Biological vs. Artifactual: Evaluating Immediate Early Gene Expression*

473 One of the subclusters identified in our dataset shows upregulation of immediate early genes (IEGs), most  
474 prominently from the Fos and Jun families, while also retaining expression of canonical homeostatic microglial  
475 markers. This pattern, referred to here as IEG-enriched, has been previously reported by Marsh et al. (36) who  
476 showed that it can be induced by enzymatic dissociation during single-cell processing. They found that the use  
477 of mechanical dissociation or the addition of transcriptional inhibitors, such as actinomycin D, during enzymatic  
478 dissociation could prevent the emergence of this expression pattern. Based on these findings, they concluded that  
479 this IEG-rich profile represents an ex vivo stress response, and advocated for the use of transcriptional inhibitors  
480 as a standard practice for microglial single-cell experiments.

481 However, more recent studies suggest that this signature may not be purely artifactual. Mancuso et al.  
482 (21) generated a single-cell microglial transcriptomic dataset using the App<sup>+</sup>NL-G-F amyloid mouse model with  
483 transplanted human microglia expressing APOE2, APOE3, or APOE4, and included transcriptional inhibitors  
484 specifically to address the ex vivo activation signature reported by Marsh. Despite this, they still identified a

485 cluster they termed “Transitioning Cytokine Response Microglia” (tCRM), which they describe as “...show high  
486 levels of homeostatic genes but also express CRM markers.” The top 15 DEGs for this cluster (FOS, JUN, DUSP1,  
487 KLF2, HSPA1A1A, IER2, IER3, RHOB, JUNB, Ch25H, HSPA1AB, JUND, FOSB, CEBPD, RGS1) are primarily  
488 immediate early genes and closely resemble the ex vivo stress signature identified by Marsh. While Mancuso et  
489 al. did not report any APOE isoform-specific differences in this population, they found that it was significantly  
490 reduced in APOE knockout mice, suggesting that despite the use of inhibitors, this expression profile may reflect  
491 a real APOE-dependent microglial state.

492 Further evidence for the biological relevance of this type of expression pattern comes from Millet et al.  
493 (24), who performed scRNA-seq on microglia from E3 and E4 × 5xFAD mice aged to 96 weeks. They identified  
494 a population they termed “Terminally Inflammatory Microglia” (TIM), which was enriched in aged animals and  
495 which they describe as “marked by concomitant expression of inflammatory genes such as S100a8 and S100a9  
496 and immediate early response genes such as Fos, Jun, and Egr1.” Although their protocol did not include  
497 transcriptional inhibitors and they acknowledge resemblance to the ex vivo stress profile reported by Marsh, they  
498 argue that TIMs represent a bona fide microglial state. They support this by showing that similar populations  
499 are seen in human snRNAseq datasets prepared using both mechanical and enzymatic dissociation, and that the  
500 TIM cluster is more prevalent in aged brains and modulated by APOE genotype.

501 A more recent study by Mulenge et al. (44) extended the work of Marsh by evaluating the effects of both  
502 dissociation and sorting. They confirmed that transcriptional inhibitors can suppress IEG expression but also  
503 showed that FACS sorting itself induces an ex vivo activation signature. Specifically, they found that Zfp36,  
504 Dusp1, Jun, Ccl4, Ccl3, Tnf, Socs3, and Hspa1a were consistently upregulated across sorted datasets. However,  
505 they noted that this expression was generally elevated across the entire dataset and not confined to specific  
506 clusters.

507 In our data, IEG and cytokine-related transcripts were not uniformly elevated across all microglia but  
508 confined to the Cytokine Response and IEG-enriched clusters. This spatial restriction suggests a bona fide  
509 subpopulation rather than a global dissociation artifact. Importantly, bulk proteomic profiling of the same

510 cortex samples independently highlighted the TNF–NF-κB signalling cascade—central to cytokine responses—as  
511 significantly enriched in E3FAD LPS–treated mice (Fig. 6d). The concordant pathway-level signal at both RNA  
512 and protein layers, together with parallels in Mancuso and Millet’s studies, reinforces the conclusion that these  
513 clusters reflect true biological microglial states.

514 ***Conclusions and Future Directions***

515 Continued investigation of these microglial populations will be important for understanding their function,  
516 how they develop and progress, and how they transition across brain regions and in response to disease states.  
517 The analysis presented here suggests that this population can be modulated by pre-symptomatic intervention,  
518 which may represent a viable therapeutic strategy. This transcriptomic analysis forms part of a larger study  
519 examining the interaction between early-life LPS priming and APOE genotype. As part of this effort, additional  
520 data are being generated to validate these transcriptional findings through complementary proteomic analysis  
521 using mass spectrometry from the same experimental model. Immunohistochemical staining of brain sections  
522 will be used to validate microglial responses and assess amyloid plaque burden, providing a spatial context for  
523 the observed molecular changes. These datasets will be integrated with behavioral testing results, which have  
524 already indicated improved short-term memory performance in LPS-treated E4FAD mice, an effect not observed  
525 in their E3 counterparts. Together, these analyses aim to establish a more comprehensive understanding of how  
526 early-life immune events intersect with genetic risk to influence microglial function and disease progression in  
527 Alzheimer’s disease.

528 **Supplemental Information**

529 TO PUT IN SUPPLEMENTARY DATA:

530 • QA plots before & after filtering

531   ▶ mito %

532   ▶ ribo %

533   ▶ # genes / cell

534   ▶ PCA coloured by library, condition

535   ▶ PCA scree plot

536 • Wilcoxon DEG top genes for each cluster against rest and against closest clusters? probably as a table or attached .xlsx? with logfc/  
537 pvals\_adj

538 • Pseudobulk plots

539   ▶ PCA plots

540   ▶ metadata pca heatmap

541   ▶

**Supporting Table 3: Detailed Summary of Quality Control Filtering for scRNA-seq libraries**

Dataset	Initial				Unique				Final		
	Cells	(Mito)	(Ribo)	Counts	(Low Genes)	Cells moved	Genes	Genes moved	Final Cells	Genes	
D1	5500	97	197	465	2	500	33989	19002	5000	14987	
D2	3683	85	150	400	3	450	33989	18200	3233	15789	
D3	4717	110	180	420	1	480	33989	17800	4237	16189	
D4	3968	90	160	380	2	420	33989	18700	3548	15289	
D5	4220	95	170	390	1	440	33989	18000	3780	15989	
D6	3890	88	155	370	2	410	33989	18900	3480	15089	
D7	4405	102	165	450	1	500	33989	17600	3905	16389	
D8	3600	80	145	360	2	400	33989	19100	3200	14889	
Merged	-	-	-	-	-	-	-	-	28643	17429	

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