Age-specific activation of microglial genes and crosstalk between glial cells modulate Trem2-dependent immune response in Alzheimer's disease

Haoyang Zhou

Abstract

Alzheimer's disease (AD) is a neurodegenerative disease that increases its prevalence with age. AD is characterized by a progressive loss of cognitive function, leading to dementia. The conventional pathological hallmarks of AD include extracellular deposition of amyloid plaque, intraneuronal aggregation of neurofibrillary tangles (NFT), and inflammation. Recent studies have confirmed that immune responses play an important role in the development and progression of AD, such as sustained inflammation within the brain. Furthermore, single-cell RNA-sequencing (RNA-seq) studies of the AD mouse model identified the disease-associated microglia (DAM) population during AD progression, where the pro-inflammatory activity was mediated by Trem2 cascade activation. However, the specific time points and precise mechanisms of DAM activation remain largely unknown, resulting in limited identification of drug targets. To better understand the molecular pathology of AD and potentially develop novel drug targets based on neural immunotherapy, we compared the single nuclei RNA-seq data in wildtype (WT) and Trem2 knock-out (Trem2 KO) mouse models and discovered the age-dependent activation of Trem2 targeted immune-related genes. We showed that Trem2 affected DAM activation at an early stage of AD progression (4-8 months) rather than the late stage (12-16 months). We demonstrate that potential AD immunotherapy could be developed by targeting immune genes at early stages; while targeting immune genes at late stages may cause higher off-target rates, given the obscure boundary between DAM and other homeostatic microglia. In addition, we found that other glial cell types, such as oligodendrocytes and astrocytes, also acquired disease-specific cell populations and expression profiles, indicating the possible crosstalk between glia involved in Trem2-dependent immune response. Our findings revealed the existence of disease-associated glial cell populations outside of DAM and recommend applying immunotherapy in the early stage of AD.

Keywords: Alzheimer's Disease, disease-associated microglia, immunotherapy, single-cell sequencing, transcriptomics, genomics, Trem2

CONTENTS

1 Introduction

Alzheimer's disease (AD) is an irreversible neurodegenerative disease that is prevalent in 1 in 10 people over 65 years old and contributes to 100,000 deaths each year. The prevalence of AD increases as people age with around 0.6% of the population aged between 65 and 69 and, on the contrary, 8.4% after 85 years old (Hebert, 1995; Long & Holtzman, 2019; Drew, 2021; Alzheimer's Association, 2021). The primary symptom of AD is cognitive function loss which eventually leads to dementia (CDC, 2020). There are no disease-modifying treatments of AD so far, and most of the currently approved treatments approved by the US Food and Drug Association (FDA) are limited to the alleviation of symptoms, not the root cause of AD. Conventional anti-AD drugs usually are developed in the form of cholinesterase inhibitors or N-methyl-D-aspartate (NMDA) antagonists (such as Donepezil, Rivastigmine, Memantine, and Galantamine), which only mildly improve the cognitive defects in patients but cannot delay disease progression (Yiannopoulou & Papageorgiou, 2013).

In order to find disease-modifying therapeutics for AD, it is thus a necessity to explore the molecular pathways involved in AD. The two canonical hallmarks of AD are the accumulation of amyloid plaque and neurofibrillary tangles (NFT) between and within neurons, which are composed of aggregated Amyloid-β and Tau proteins respectively (**Figure 1**, red and blue lines). For the past few decades, most new therapeutic developments have been relying on these two hallmark features of AD (Drew, 2021). However, very little success has been achieved, and there are still few disease-modifying effects verified from AD patient studies (Tampi et al., 2021). Currently, only Aducanumab has been approved to target the cause of AD by removing Aβ Plaques (Commissioner, 2021; US Department of Health, 2021; "FDA grants accelerated approval for alzheimer's drug," 2021). However, the efficacy of Aducanumab is being questioned (the EMERGE trial NCT02484547; the ENGAGE trial NCT02477800). In both phase 3 trials, Aducanumab failed to achieve statistical significance in low doses compared with the placebo (P value 0.09 and 0.22), and in the ENGAGE trial, Aducanumab at high doses also showed no significance compared with the placebo (P value 0.83) (Knopman et al., 2021). Further, there is an unclear relationship between Aβ removal and cognitive improvements thus the clinical benefits of Aducanumab remain unclear (Congdon & Sigurdsson, 2018; Sabbagh & Cummings, 2020; Tampi et al., 2021). Though tau has shown more correlation with AD severity compared with Aβ, non-immunotherapy drugs like Tideglusib and Epothilone D have largely been discontinued in the research process due

to toxicity or lack of efficacy, while immunotherapy-related research like AADvac1 and RG7345 has mostly been proven successful in mice but has yet to be tested in humans (Sayas, 2020).

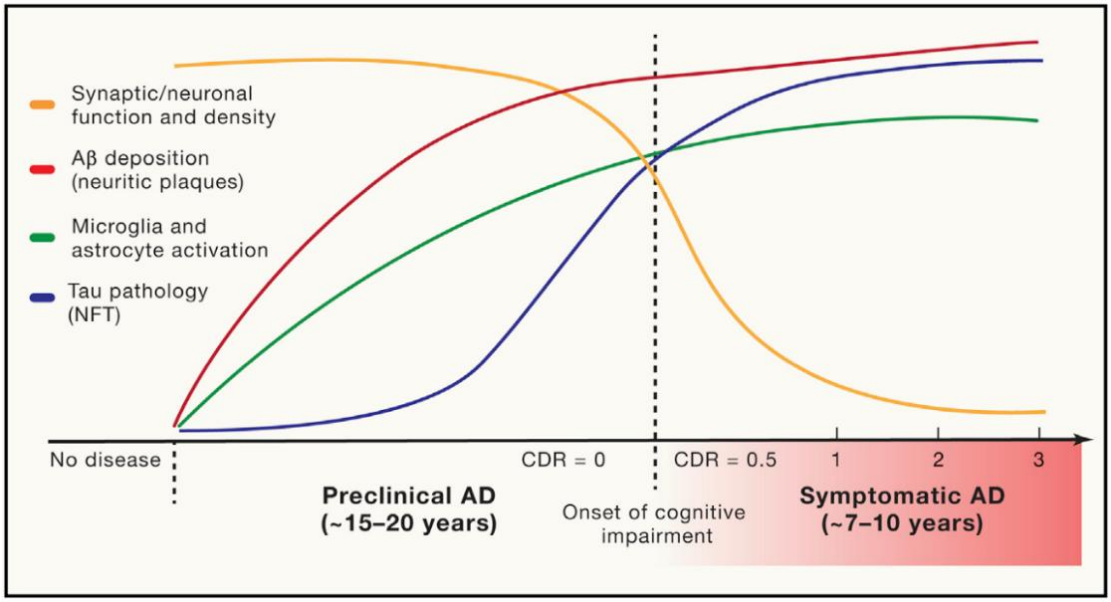


Figure 1. **Major Pathopsychological Progression in Relation to Clinical Progression of AD.** Cited from Long and Holtzman, 2019 "Alzheimer Disease: An Update on Pathobiology and Treatment Strategies"; The timing of major pathophysiological events in relation to clinical course. levels of Aβ (red line) and activated microglia (green) showed a more significant increase at an earlier stage compared to tau (blue). Cognitive abilities represented by synaptic/neuronal function (yellow line), on the other hand, start to decrease approximately around when the level of tau was seen to significantly increase.

For the past few decades, neuroinflammation, a completely new hallmark of AD, has been extensively studied, and emerging evidence has suggested that it is a key pathological process as a novel target for AD treatments. In particular, microglia, one cell type of neuroglial, is responsible for the immune response in the brain, removing Aβ and damaged brain cells via phagocytosis as (Roger et al., 1996). However, chronic stimulation of microglia by Aβ or other misfolded proteins may lead to prolonged inflammation, which is harmful to the neurons (**Figure 1**, green line) (Bachiller et al., 2018, Long & Holtzman, 2019). Post-mortem studies have revealed that young patients who experienced systemic inflammation have similar morphological changes of microglia to people experiencing dementia. In addition, asymptomatic patients show no relevance between such microglial morphological changes and Aβ load, indicating neuroinflammation may be an independent or even prerequisite

pathological event to the amyloid plaque for AD (Streit et al., 2009; Leng & Edison, 2020). With the recent development of sequencing technology, genome-wide analyses have shown that the most differentially expressed genes between AD and control are immune genes rather than neural genes (Landel et al., 2014). Furthermore, single-cell transcriptome studies have revealed that these AD-related immune response genes, also termed disease-associated microglia (DAM) genes, can be activated by a key immunological gene, Trem2 (Triggering Receptor Expressed on Myeloid Cells 2), expressed in disease-specific populations of microglia (**Figure 2**) (Landel et al.; Keren-Shaul et al., 2017). Several mutations of Trem2, such as R47H and R62H, have been identified to increase late-onset AD risk by 2 to 4 folds (Guerreiro et al., 2013; Gratuze et al., 2018). Consistently, 5x-MITRG AD mouse model injected with isogenic human microglia that differentiated from CRISPR-modified *TREM2*-knockout induced iPSCs showed an increase in Aβ load while preventing microglia to obtain DAM signatures (McQuad et al., 2020). Similarly, Trem2 KO exacerbates the AD phenotypes in 5xFAD mice, causing increased Aβ load and neuron loss. However, Trem2 KO has also been shown to attenuate the expression of inflammatory cytokine transcripts in the same 5xFAD model (Wang et al., 2015). It is still intriguing the precise role of DAM in AD and if there is a gene expression profile change in DAM as the AD model ages.

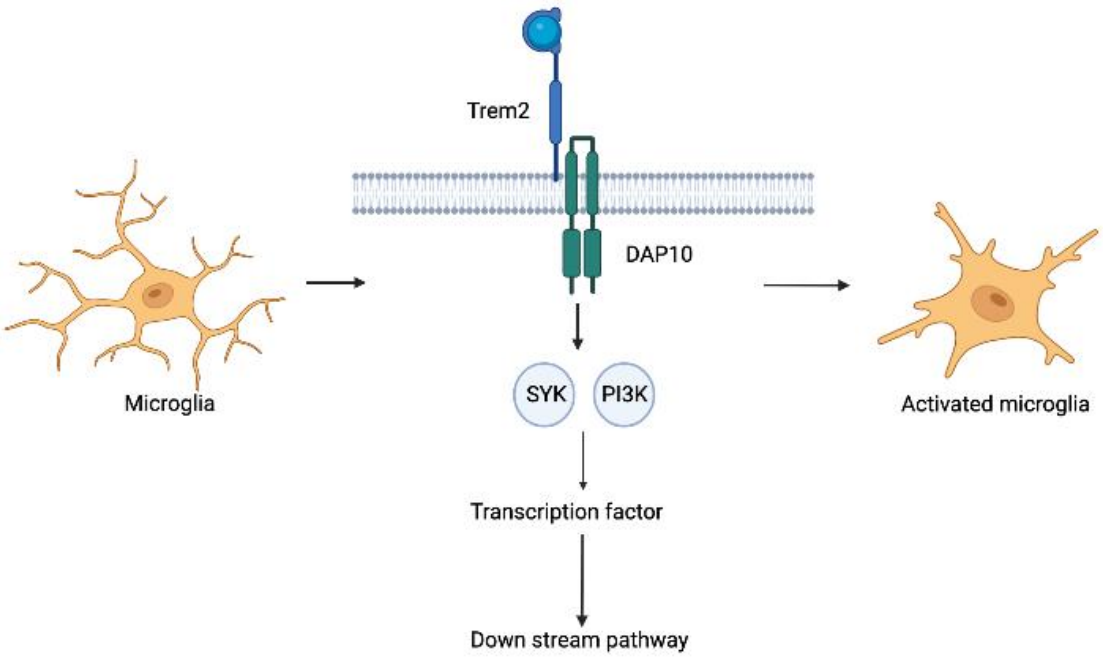


Figure 2. **The TREM2-regulated pathway in microglia activation.** Microglia can be activated by the Trem2 and DAP10 complex binding to specific ligands that induces SYK and PI3K phosphorylation, which then triggers downstream pathways. leading to microglia activation. Dysregulation in this signaling is one of the hallmarks of AD.

Nevertheless, more and more studies explored AD treatment development with a specific focus on immune genes and immune cell populations, such as microglia and macrophages in the brain (Liddelow et al., 2017). This raises significant attention to immunotherapy for AD. However, non-steroid anti-inflammatory drugs (NSAIDS) (Ibuprofen, Simvastatin, Azeliragon, Pioglitazone), which generally inhibit inflammation, failed during phase 3 of the trial due to the lack of efficacy, while a few other drugs are undergoing phase 2 (Dong et al., 2019). We propose that immunotherapy, used in conjunction with other treatments, would be a promising therapeutic strategy for AD. However, a major roadblock in identifying prevalent immune-related drug targets is the detailed understanding of when and where DAM and neuroinflammation are engaged in AD.

Here, we focused on gene expression activated by Trem2 since this is the most well-studied responsive immune-related gene in AD patients. We utilized bulk RNA-seq and single-cell RNA-seq to analyze the effect of Trem2 KO on the cell population. We found that Trem2-dependent or independent gene activation is age-specific. Through analysis of single-nuclei RNA-seq data, we found a disease-specific population in early-stage rather than late-stage during disease development, thus we concluded that AD treatment should be developed to target immune genes induced in the early stage to improve efficacy and avoid the off-target effect. Moreover, we found that other glial cells, such as oligodendrocytes in the early stage and astrocytes in the late stage, were also acquired in disease-specific cell population and gene expression, indicating that the crosstalk among glial cells may be involved in Trem2-dependent immune response. In addition, novel sets of disease-associated genes specific to oligodendrocytes and astrocytes were discovered in AD or AD-like pathogenesis, and they could be used as novel drug targets in AD treatment.

2 Methods

2.1 Data collection

GSE168137 (Forner et al., 2021) was used for bulk RNA-seq data analysis to understand the specific genes and associated pathways of AD. Samples were collected from the hippocampus and the cortex tissue in 5xFAD mice and wildtypes at 4, 8, 12, and 18 months old. Datawere aligned to mouse references mm10 and gene expressions were normalized to TPM (Transcripts per Million mapped reads) (Forner et al., 2021). GSE140510 and

GSE140399 (Zhou et al., 2020) were analyzed for single-cell RNA-seq to show details in microglia and other glial populations. GSE140510 was from cortex in mice at 7 months old (considered as early stage in AD) and GSE140399 was from mice at 15 months old (late stage), with each containing 4 genotypes, including wildtype, 5xFAD, *Trem2* KO, and 5xFAD; *Trem2* KO. The UMI count matrix was collected for downstream analysis (Zhou et al., 2020).

2.2 Data preprocessing

In bulk RNA-seq data, lowly expressed genes were filtered out and gene expression matrices were finalized by creating a threshold of TPM $\geq$ 1 and expressed in at least 5 replicates. Further, the data were quantile normalized using the R package, Limma (Ritchie et al.., 2015). In single-cell RNA-seq data, samples with high mitochondria percentage and low number of expressed genes were filtered out. This analysis was conducted by R package, Seurat (Hao et al., 2021).

2.3 Principal component analysis (PCA)

PCA is used for dimension reduction to isolate the most important principal components that account for the highest variance. PCA is conducted here to visualize batch effects and technical effects in RNA-seq experiments. Bulk gene expression matrix was first log2 transformed and fed into R function prcomp, with center equals to true and scale equals to false.

2.4 Differential gene expression analysis

In order to understand the differentially expressed genes in AD, read counts of genes are compared between disease samples and wild type. A gene is declared differentially expressed when the difference is significant (FDR<0.05). ExactTest, from EdgeR package (Robinson et al., 2010), was used to conduct differential gene expression analysis.

2.5 Time course differential analysis

Time course differential analysis across brain regions, time points or genotypes was done by masigPro (Nueda et al., 2014). Rsq=0.7 and T.fit α=0.05 were defined to cluster differentially expressed genes and isolate AD-related genes, brain region-specific genes, and aging-related genes.

2.6 Gene ontology analysis

Gene ontology is an effort to unify the terminology of gene function. To understand the specific pathway of the upregulated and downregulated genes, gene lists were fed into Metascape (Zhou et al., 2019) to conduct gene ontology analysis with input species as mouse. Enriched terms were selected based on hypergeometric tests (BH-corrected p-value<0.05).

2.7 Single-cell clustering

Single-cell clustering was conducted by R package, Seurat (Hao et al, 2021). The dimension reduction was conducted with the UMAP algorithm to observe the cell type clustering based on their gene markers. Mixed population and doublets were removed and cell type-specific markers and differential markers between treatments/genotypes were found.

2.8 Normalization for relative enrichment

Relative enrichment of each cell type within a cluster is calculated with the following equation.

$$\text{Relative enrichment} = \frac{\text{Cell count(given cell type within a cluster)}}{\text{Cell count(respective genotype)} \times \text{Cell count(respective cell type)}}$$

3 Results

3.1 Upregulation of immune response genes in an early stage of AD progression

In order to understand the temporal pattern of neuroinflammation in AD, we observed brain regions and ages that are most affected by AD-like pathology. We collected published bulk RNA-seq samples from the cortex and hippocampus in both 5xFAD and WT mice at the age of 4, 8, 12, and 18 months respectively (Zhou et al., 2020). To verify the quality of the data, PCA was performed. Data from the hippocampus were clearly separated by time or age with PC1 accounting for 28.8% of variances. The data was also separated by genotypes, WT and FAD, shown in both PC1 and PC2, which accounted for 41.1% of variances in total (**Figure 3A**). In addition, the differences between 5xFAD and WT became more pronounced when comparing the early stage (4 and 8 months) with the late stage (12 and 18 months)

(**Figure 3A**).

Similarly, we found that data from the cortex samples could also be separated by PC1 (30.6% variances), but with no clear PC indicating the separation based on genotypes (**Figure 3B**). Then, we examined the weight's contribution of each gene in the cortex samples and found that PC4 (5.87% variances), containing immune-related genes, contributes mostly to the separation between 5xFAD and WT in the cortex.

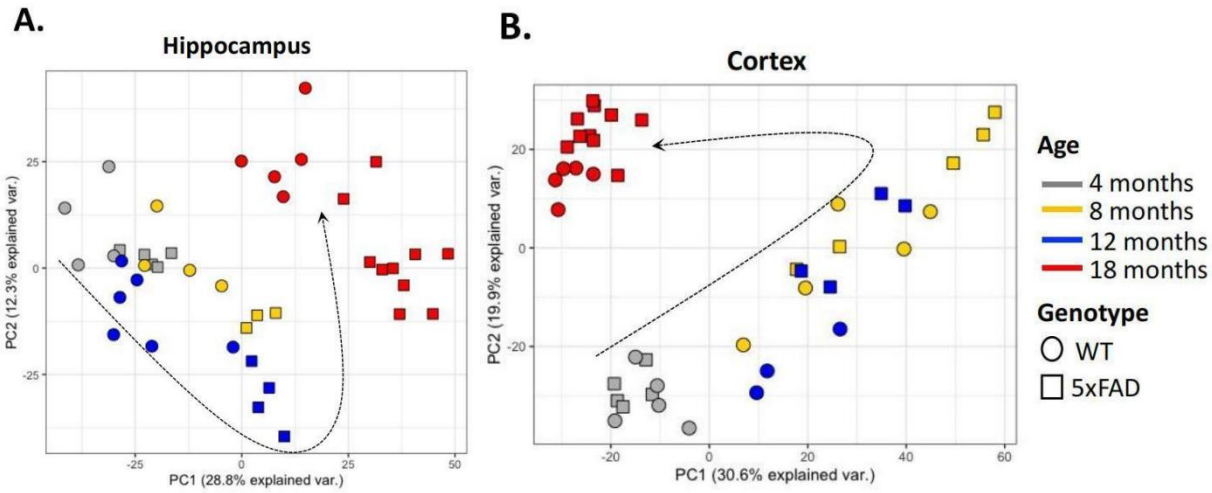


Figure 3. **PCA of bulk RNA-seq data from 5xFAD and WT mice.** PCA of RNA-seq data from 5xFAD and WT mice in the hippocampus (A) and cortex (B) across 4 time points (or ages). The time point was represented by different colors and shapes denoting the genotype of mice.

In all, the results showed that normal aging has a larger impact than AD-like pathology in brain regions, and aging could be considered a significant factor driving individual transcriptome variance.

To observe transcriptomic differences between FAD and WT with regard to age, we performed differential gene expression analysis in the hippocampus and cortex between these two genotypes at each time point. We determined the number of significantly upregulated genes in the 5xFAD and WT with respect to time. Finally, we overlapped the differential genes to a list of DAM genes (Sobue et al., 2021) to observe how many immune-related genes were activated in terms of aging with/without AD-like pathology. In both hippocampus and cortex, there were drastically more genes that are being upregulated in the 5xFAD model compared to the WT (**Supplementary Table 1**). We found an increasing number of upregulated genes in 5xFAD samples along with aging in both hippocampus and cortex (**Supplementary Table 1**). We also observed that the hippocampus was affected at a larger

magnitude than the cortex, with over doubling the genes upregulated in all 4 time points in 5xFAD samples (**Supplementary Table 1**). Then, the genes upregulated in both the hippocampus and cortex showed significant overlap with disease-associated microglia (DAM) genes, especially in the early to mid-stage. About 48% and 19% of all upregulated genes in the hippocampus of 5xFAD mice are DAM genes at month 4 and month 8 respectively. And in the cortex, 27% and 13% of all genes upregulated in 5xFAD mice are DAM genes at months 8 and months 12 (**Figure 4A and 4B**).

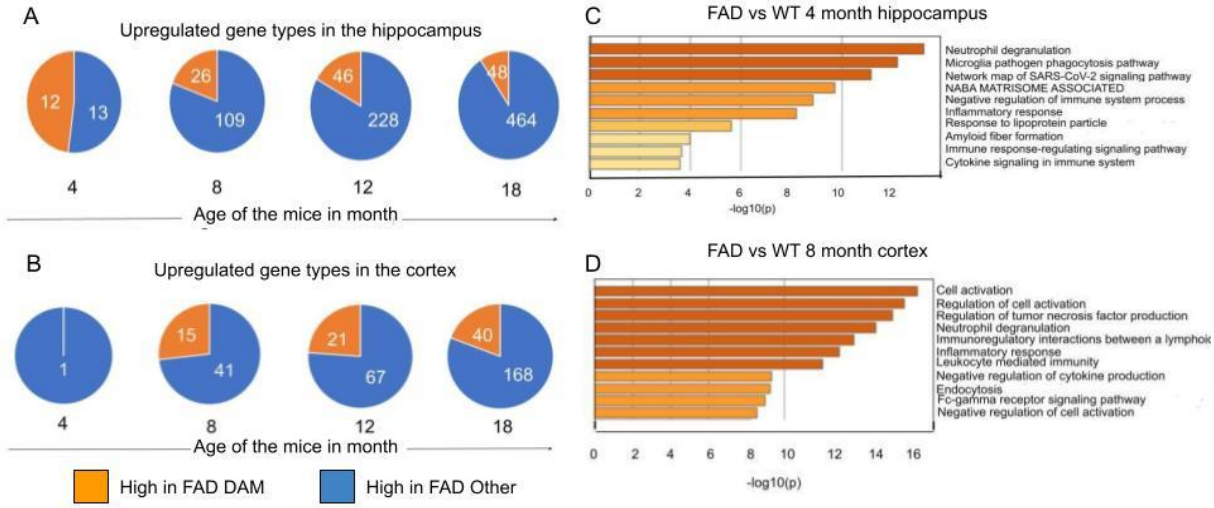


Figure 4. **Upregulated genes in FAD and relevant GO terms. (A & B)** Summary of differentially expressed genes selected with FDR<0.05 between 5xFAD and WT samples in the hippocampus (upper panel) and cortex (bottom panel), listed with the number of upregulated genes in each genotype and of which how many DAM genes at each time point. **(C & D)** Examples GO term of differentially expressed genes of 4 months in the Hippocampus (Upper panel) and 8 months in the cortex (lower panel).

In order to understand different variables driving these gene expression changes, time-course analysis was conducted to observe changes in AD-like pathology in both hippocampus and cortex. K-means clustering was used to further classify differentially expressed genes which resulted in 8 clusters based on their expression profiles (**Figure 5A**), Among them, we focused on gene clusters that were specifically affected by aging, brain region, and FAD effect respectively. In clusters 1, 3, and 6, we observed a consistent gene regulation across 4 genotypes in 2 brain regions, suggesting these genes were only affected by aging but not AD-like pathology or brain regions (**Figure 5A**). GO terms associated with these clusters included RNA metabolic process, cellular component morphogenesis, synaptic organization, chromatin

organization, histone modification, and plasma membrane bounded cell morphogenesis **(Figure 5B)**. Moreover, cluster 2 and cluster 8 reflected the impacts of brain regions (**Figure 5A**). GO terms associated with these clusters were cell junction organization, synaptic signaling, neuron projection metamorphosis, and neuronal death, behavior, and blood circulation.

Importantly, we found only cluster 5 showed gene expression profiles affected by AD-like pathology in both hippocampus and cortex (**Figure 5A**). Three hundred and two genes included in this cluster were involved in pathways like immune effector process, regulation of cytokine production, myeloid leukocyte activation, response to wounding, phagocytosis, and response to the virus (**Figure 5B**). Comparing cluster 5 (representing FAD effect) with cluster 1 (representing aging effect), we found that GO terms of FAD driving genes have a more significant focus on the immune response, signifying a strong association between AD pathology and immune response.

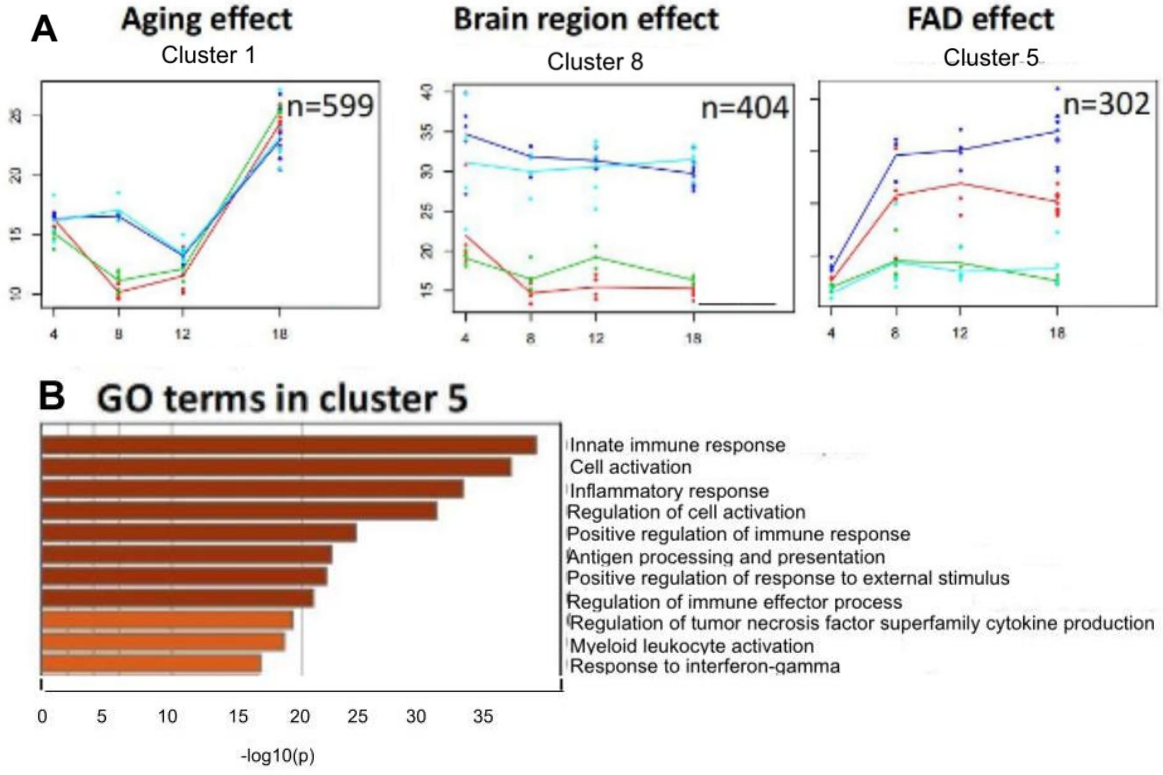
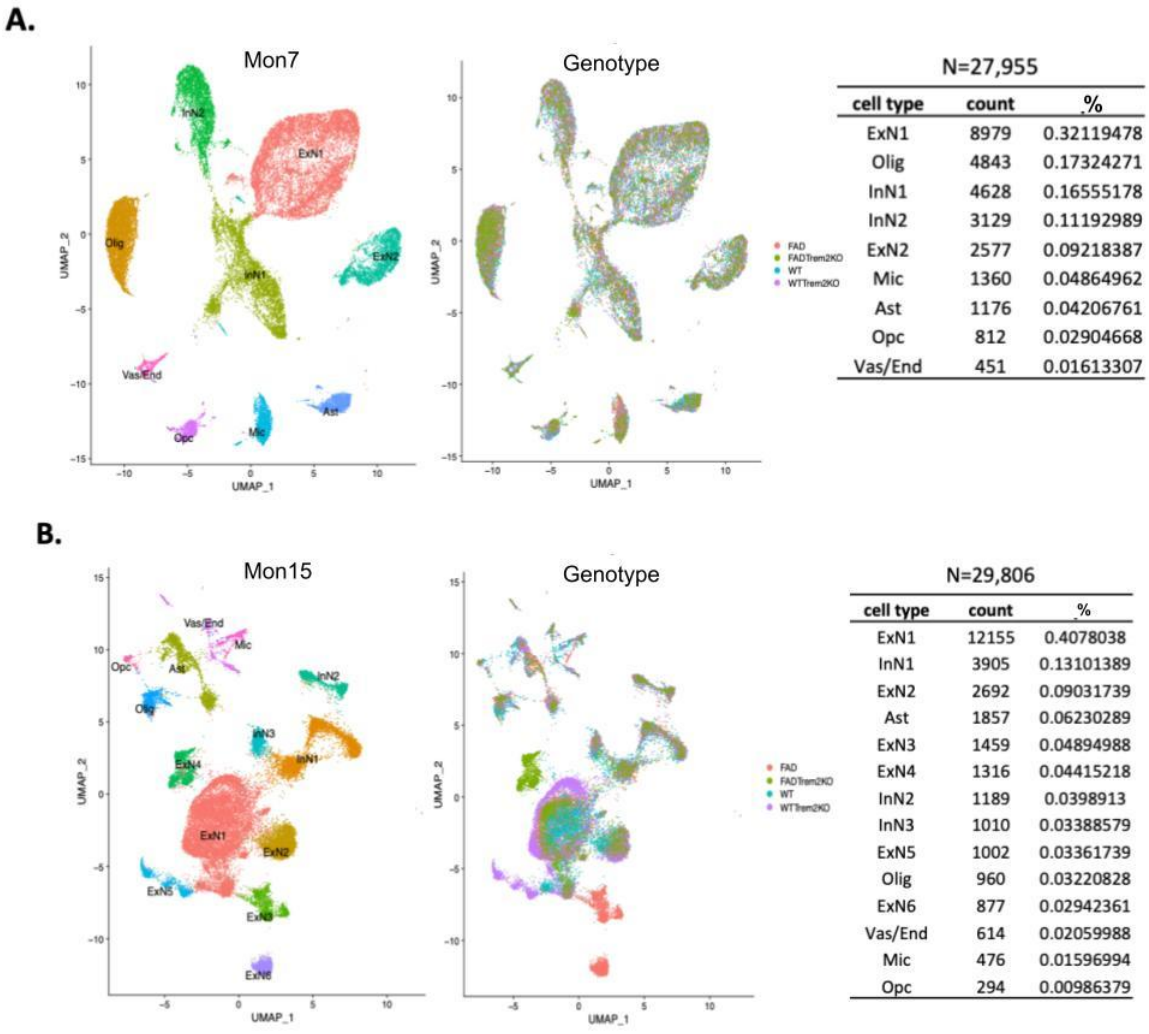


Figure 5. **Time course analysis illustrated expression patterns specific to aging, brain region and FAD effects. (A)** The vertical axis represents log2(TPM+1), and the horizontal axis represents age; the brain region and genotypes are separated by color. **(B)** GO terms associated with genes in cluster 5 were highlighted and ranked by significance level.

Above all, through comparative bulk transcriptomic analyses from the mouse model, we pinpointed the immune-related genes and associated immunological response pathways during AD progression. We have shown that DAM genes are highly upregulated in the early stage of AD progression, while their proportions decrease as age increases. While such gene expression patterns remain consistent in the cortex and the hippocampus, the effect on the hippocampus can be observed at a relatively earlier time point.

3.2 Trem2-dependent enrichment of microglia in an early stage during disease progression



N=27,955

cell type	count	%
ExN1	8979	0.32119478
Olig	4843	0.17324271
InN1	4628	0.16555178
InN2	3129	0.11192989
ExN2	2577	0.09218387
Mic	1360	0.04864962
Ast	1176	0.04206761
Opc	812	0.02904668
Vas/End	451	0.01613307

N=29,806

cell type	count	%
ExN1	12155	0.4078038
InN1	3905	0.13101389
ExN2	2692	0.09031739
Ast	1857	0.06230289
ExN3	1459	0.04894988
ExN4	1316	0.04415218
InN2	1189	0.0398913
InN3	1010	0.03388579
ExN5	1002	0.03361739
Olig	960	0.03220828
ExN6	877	0.02942361
Vas/End	614	0.02059988
Mic	476	0.01596994
Opc	294	0.00986379

Figure 6. **Cell clustering and cell count of single-nuclei RNA-seq data** Cell type clustering based upon single-nuclei RNA-seq data at the early stage, 7 months **(A)** and late stage, 15 months **(B)**. Nuclei were labeled by cell type and genotypes in UMAP, number and proportion of nuclei for each cell type

were listed. (ExN - excitatory neuron, InN - inhibitory neuron, Olig – oligodendrocyte, Ast – astrocyte, Mic – microglia, Opc-Oligodendrocyte progenitor cell, Vas/End – vascular and endothelial cells)

In order to recapitulate the molecular features of DAMs and how they affect different cell populations during the progression of AD, we analyzed single-nuclei RNA-seq data of mice at 7 months and 15 months to compare the transcriptomics profile changes in different cell types between two ages, with/without Trem2, a potential target to elicit protective role of microglia in AD (Zhou et al., 2020; Ulland & Colonna, 2018). First of all, to better visualize the characteristics of the different cell types, the UMAP algorithm was applied to reduce dimensions on the single nuclei data, and cell clusters were generated using the data at 7 months and 15 months respectively (**Figure 6**). By comparing similar numbers of recovered nuclei, we found that there was more neuron diversity (more neuronal clusters) in 15 months than 7 months. Further, we observed lower proportions of glial cells at 15 months compared to the 7 months data. We noted that oligodendrocytes were enriched at a much higher percentage at 7 months **(Figure 6 A&B)**.

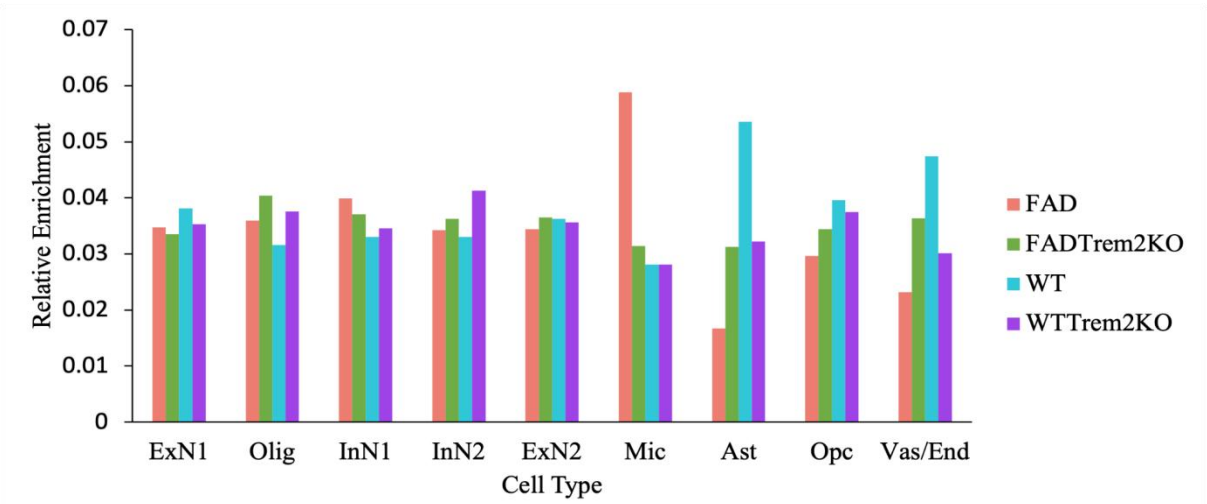


Figure 7. **Relative enrichment of different cell types among the four genotypes in the early stage** Relative enrichment Histogram of relative enrichment of each cell type in 4 genotypes in the early stage. The number of nuclei was normalized by the number of cells in the cell type and the number of cells in each genotype to obtain relative enrichment (**Methods**).

After normalization by cell type and genotype, the relative enrichment of each cell type was displayed in Figure 7. Strikingly, we found that the microglia were more specifically enriched in FAD genotype at 7 months, but not in other genotypes (WT, WT Trem2 KO, and FAD Trem2 KO) **(Figure 7)**. However, at 15 months, this enrichment was not observed and could be due to the aging effect in WT. In 15 months, both Trem2 KO, and FAD Trem2 KO

have a lower enrichment in microglia, while aged WT mice and FAD mice have similar levels of enrichment (**Supplementary Figure 1**). This could potentially indicate that microglia activation, either disease-associated or aging-associated, was dependent on Trem2. Given that Trem2 KO 5x FAD mice have accelerated AD phenotypes (Long & Haltzman, 2019), we postulate that microglia activation may not be always associated with aggravation of AD given the previously negative impact of Trem2 KO in AD, and detailed exploration of which DAM or DAM specific gene expression profile is needed (**Results Part 3**).

In contrast, other glial cells, such as astrocytes and oligodendrocytes progenitor cells (OPCs), were more enriched at 7 months and in WT, while in the oligodendrocytes, the WT seems to be more enriched at 15 months. FAD and both Trem2 KO samples have similar enrichment levels **(Figure 7 & Supplementary Figure 1)** (further discussed in **Results Part 4**).

3.3 Age-specific Trem2-dependent activation of DAM genes in glial cells and more specifically microglia population

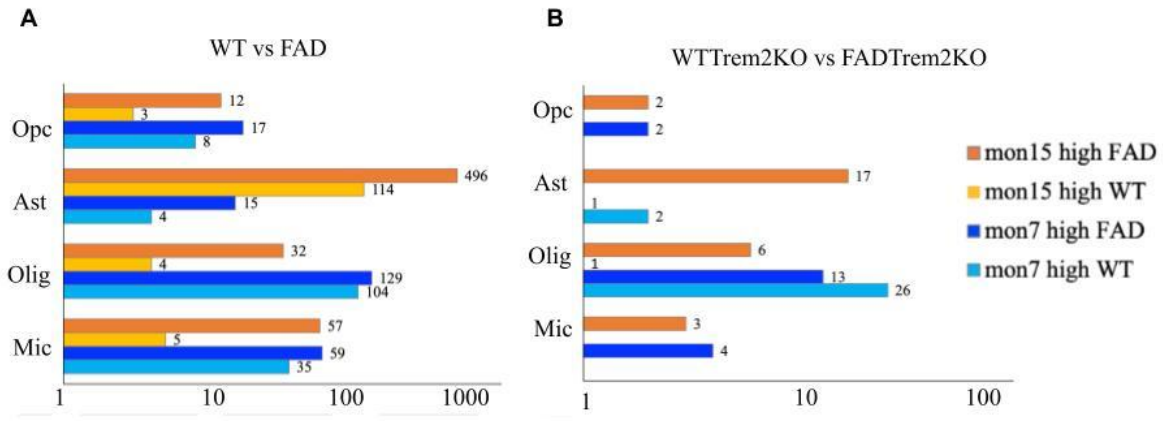


Figure 8. **Numbers of differentially expressed genes comparing WT vs FAD and FAD vs FADTrem2KO (A)** Histogram of differentially expressed genes between FAD and WT in 7 months (early stage) and 15 months (late stage) in glial cells. Genes were selected based on FDR<0.05. **(B)** Histogram of differentially expressed genes between FAD and FADTrem2 KO in 7 months (early stage) and 15 months (late stage) in glial cells. Genes were selected based on FDR<0.05.

In order to further understand the effect of Trem2 KO on gene expression in different glial cells, we performed differential expression analysis in microglia between WT and 5xFAD mouse models, as well as in other glial cell types, including astrocytes, oligodendrocytes, and

oligodendrocyte progenitor cells. Compared to 7 months, the number of differentially expressed genes in astrocytes was increased at 15 months, while the number of differentially expressed genes in oligodendrocytes and microglia was decreased **(Figure 8A)**. When comparing the gene expression profile of Trem2 KO samples in both the WT and 5xFAD model to the non-KO counterparts in Figure 6A, we observed a significant decrease in the number of differentially expressed genes across all types of glia, showing the significant effect of Trem2 on the glia transcriptomes **(Figure 8B)**. These data suggested that Trem2 was essential for the activation of DAM or DAM-related gene expression profiles.

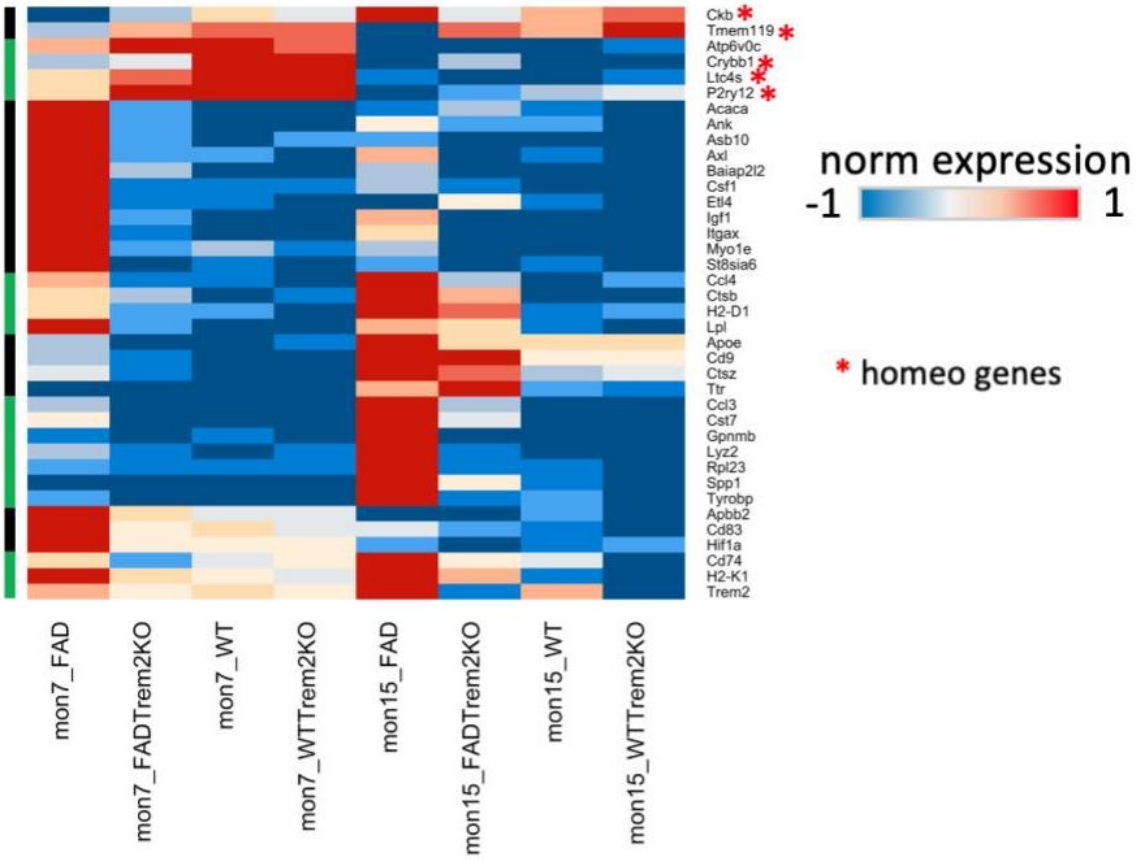


Figure 9. **Heatmap of microglia** Heatmap of gene expression of 38 genes that were differentially expressed between FAD and WT and known as DAM or homeostatic microglia markers. K-means clustering was performed to group them based on expression patterns across genotypes and time points, labeled on the left side of the heatmap. Gene expression TPM was the first quantile normalized and then transformed between -1 and 1 (blue, low expression; red, high expression). Homeostatic microglia markers were labeled with red asterisk (*).

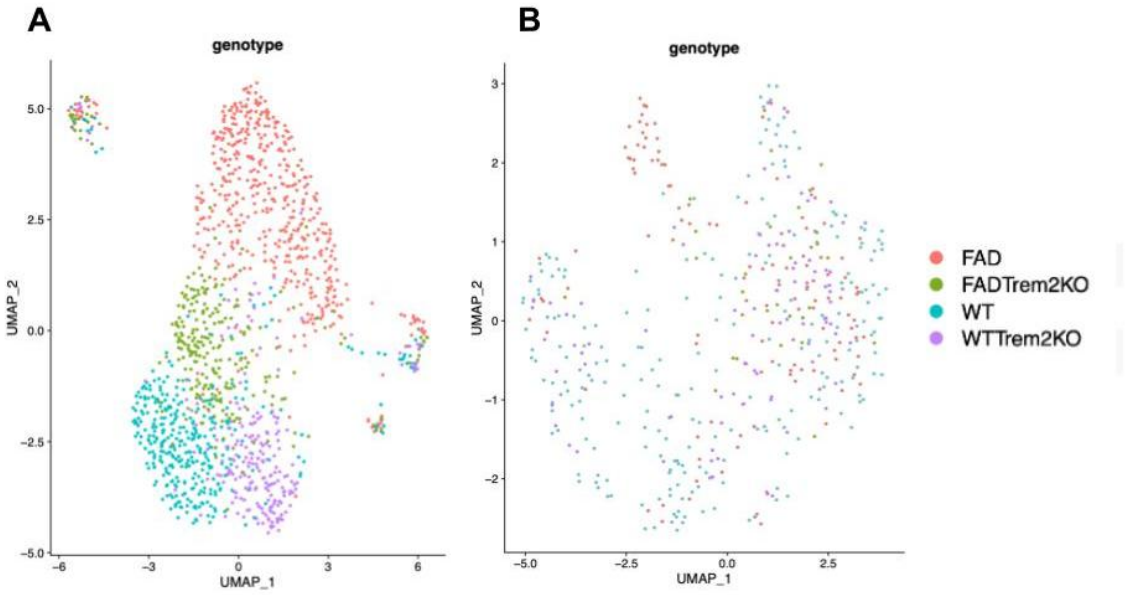


Figure 10. **Clustering of microglia** Sub Clustering of microglia nuclei in 7 months **(A)**and 15 months **(B)**. Nuclei were colored by genotypes.

To observe how specific DAM genes changed corresponding to Trem2 KO in early and late stages, we extracted differentially expressed genes between FAD and WT **(Figure 8A)** in both 7 months and 15 months, and then compared the intersection between these genes and the published DAM marker list and homeostatic microglia marker list (Sobue et al., 2021). Interestingly, out of 38 intersected genes, only 5 were homeostatic microglia markers while the rest were DAM genes **(Figure 9)**. Then we performed k-means clustering on these intersected genes and obtained 8 clusters, showing different gene expression profiles in the early and late stages among various genotypes **(Figure 9)**. Homeostatic microglia markers were only upregulated only at 7 months but not 15 months. In these homeostatic microglia cells, Trem2 had a very limited effect on these resting genes. On the other hand, most DAM genes seemed to be highly Trem2 dependent in both stages, again suggesting the potentially critical role for Trem2 in DAM activation. More specifically, the DAM genes could be separated into two distinct categories based on age **(Figure 9)**. This finding suggested DAM played different roles in the early and late stages of the disease. Unexpectedly, known markers for Trem2-independent regulation, such as Tyrobp and Lyz2, were observed in the Trem2-dependent profile of the late stage **(Figure 9)**. This raises the question of whether there existed a Trem2 independent stage of DAM (Keren-Shaul et al., 2017). Lastly, by sub-clustering on microglia nuclei, we found a disease-specific population in the early stage rather than the late stage of disease progression **(Figure 10 A and B)**. This may further raise the

possibility that DAM plays different roles at different stages of AD progression.

3.4 Crosstalk between microglia and other glia cells in modulating Trem2-dependent immune response

In addition to the microglia, we have also revealed that other glial cells, such as oligodendrocytes and astrocytes, had distinguished differential expression profiles between FAD and WT at late or early stages, which has not been extensively studied before **(Figure 7)**. Therefore, the subpopulations grouped by age and genotypes were further studied to understand how Trem2 affected the expression profiles of these genes in an age-specific manner.

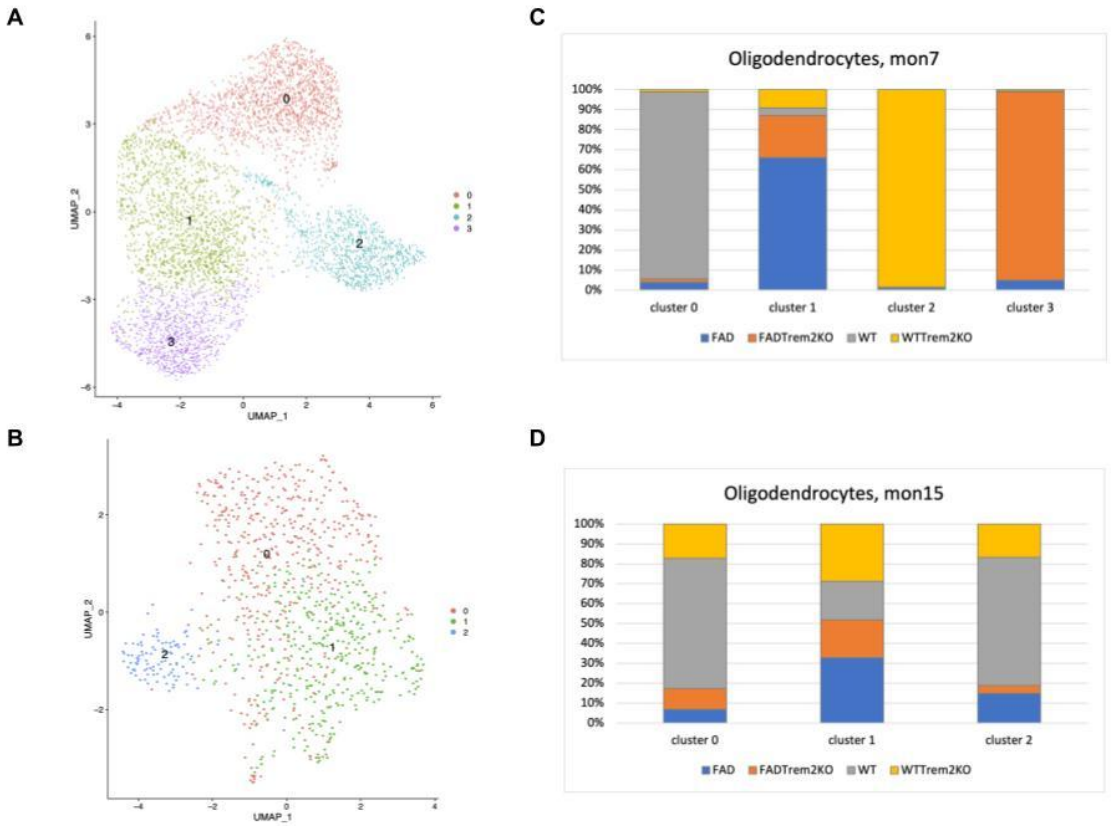


Figure 11. Sub Clustering of oligodendrocytes nuclei in 7 months **(A)** and 15 months **(B)**, with color labeled by genotypes. The proportions of each genotype in each cluster were plotted on the right with the top being month 7 **(C)** and the bottom being month 15 **(D)**.

Genotype-specific clusters in oligodendrocytes (WT Trem2 KO, FAD Trem2 KO, and 5xFAD) appeared in the early stage but not the late stage **(Figure 11 A & B)**. It further

confirmed the result in Figure 7 that more differentially expressed genes in the oligodendrocytes were found in the early stage. When observing the effect of Trem2 KO more specifically, although the Trem2 KO specific population showed up in oligodendrocytes (**Figure 11 C and D**), the differential genes between FAD and WT mice were affected by Trem2 KO minimally **(Figure 13 A)**. It indicated that the effect of Trem2 on the gene expression profile of oligodendrocytes may be mediated by other mechanisms.

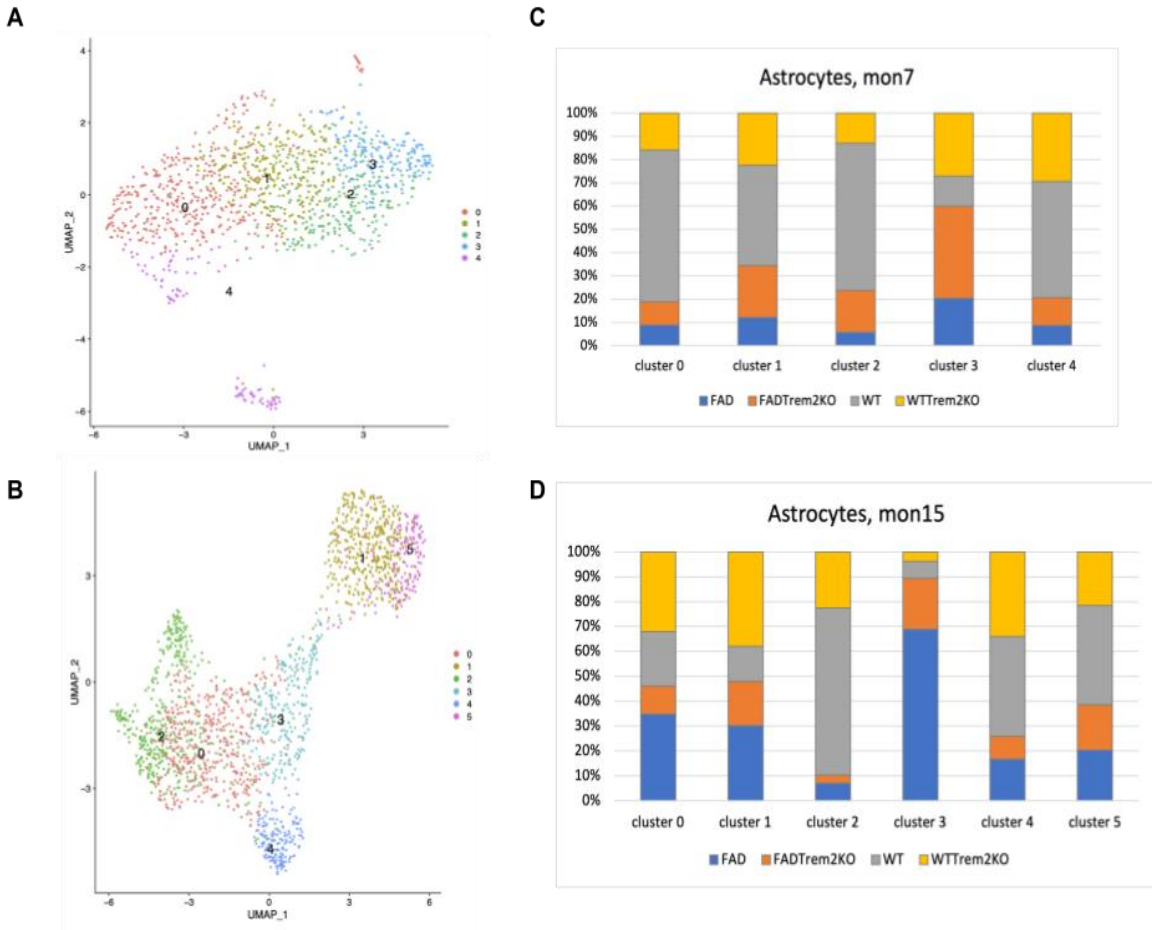


Figure 12. Sub Clustering of astrocytes nuclei in 7 months **(A)** and 15 months **(B)**, with color labeled by genotypes. The proportions of each genotype in each cluster were plotted on the right with the top being month 7 **(C)** and the bottom being month 15 **(D)**.

In contrast to oligodendrocytes, genotype-specific sub-clustering of astrocytes was shown in the late stage **(Figure 12)**, consistent with our cell enrichment analysis **(Figure 7)**. Though the effect of Trem2 on the astrocytes is limited in the early stage, the effect is more pronounced in the late stage. In contrast to microglia where Trem2 KO exhibits a closer gene expression profile to WT, Trem2 KO astrocytes align better with the FAD astrocyte population **(Figure 13 B)**. This means if we were to develop Trem2-related therapy especially

targeting late-stage microglia, its impact on the astrocytes has to be considered. Genes associated with the disease population were mostly associated with neuron development, cognition, and immune response.

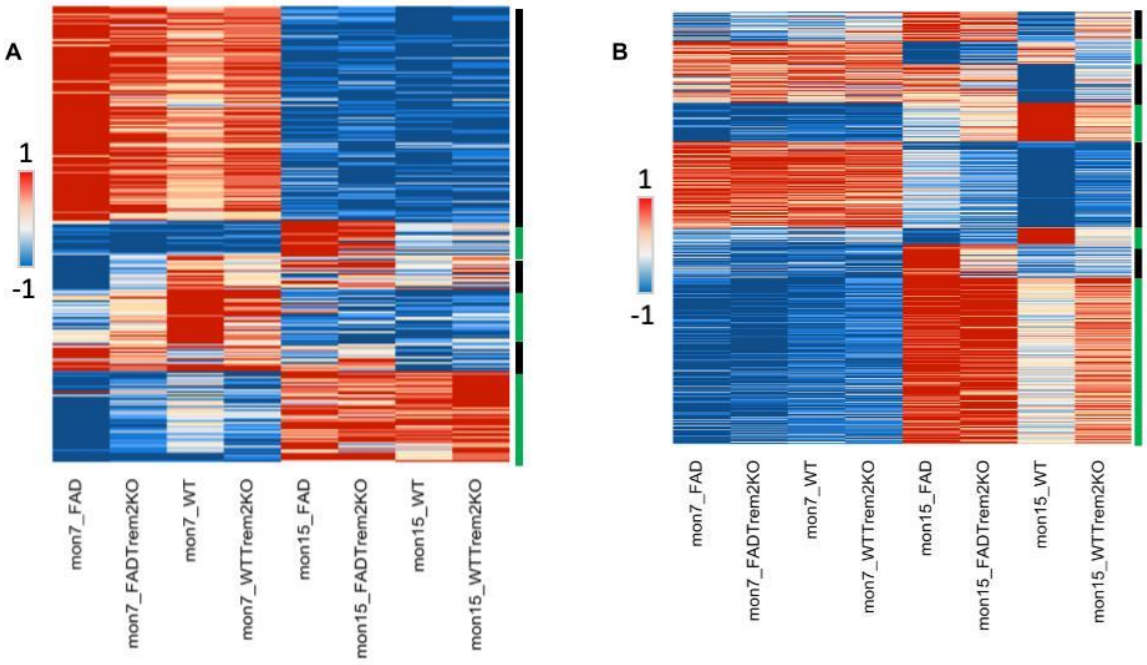


Figure 13. Expression heatmap of differentially expressed genes between FAD and WT in oligodendrocytes **(A)** and astrocytes **(B)**. K-means clustering was performed to group them based on expression patterns across genotypes and time points, labeling on the right side of the heatmap. Gene expression TPM was first quantile normalized and then transformed between -1 and 1 (blue, low expression; red, high expression).

4 Discussion

Consistent with previous studies, we showed that more DAM genes become activated with AD progression. Using bulk and single-cell analysis, we explored the characteristics of DAM further. Intriguingly, we observed that DAM showed distinct profiles in the early and late stages during AD progression. This indicated the time point-dependent mediation of DAM genes in disease-associated activation. We assumed that DAM initially plays a protective role but switches to a chronic inflammatory role later which damages neurons in AD. Furthermore, by classifying genes associated with the early and late stages, we found that more genes in the late stage are inflammation related. Such results supported the similar model proposed by Onuska (2020), in which only DAM progressively becomes more inflammation related.

Finally, we also observed that there were clearer boundaries of microglia between different genotypes in the early stage than in the late stages, suggesting that early-stage DAM had more distinct characteristics compared to WT counterparts.

In previous studies, Trem2 was identified to be a key player in DAM activation (Landel et al.; Keren-Shaul et al., 2017). Here, we confirmed further that Trem2 was highly important in DAM activation. Unexpectedly, 5xFAD Trem2 KO samples were characteristically closer to WT than their disease counterparts. However, Trem2 KO in 5xFAD mouse model is known to accelerate the AD phenotype compared with 5xFAD. One potential explanation is Trem2 is a trigger to DAM, yet the downstream effect of DAM is dependent on other regulatory factors and not Trem2. In contrast, Trem2 seems to play a different role in astrocytes, making the WT Trem2 KO samples closer in characteristic to the disease sample. This is similar to the finding that the loss of Trem2 in A1 reactive astrocytes is associated with cerebral amyloid angiopathy (Taylor et al., 2020). Further, our results showed that some previously identified Trem2 independent markers seemed Trem2 dependent in later stages.

This study provides insights into the development of novel AD treatment. Firstly, targeting earlier stages of AD might be more viable compared to later stages due to more distinct characteristics in microglia. This will avoid unnecessary off-target effects on normal aged microglia. Secondly, when developing treatment, interactions with other glial cells need to be considered. Other glial cells are also developing their own disease-specific clusters in our results, meaning they also contribute to AD. Furthermore, data from Trem2 knockout showed that other glial cells might also respond to treatments that specifically target microglia, necessitation a more precise study of cell-cell interactions.

This study has a few limitations. Firstly, the study was conducted in the mouse model. Due to physiological differences between humans and mice, it remains questionable to what extent the findings of this study translate to human subjects. Secondly, time points for single-cell sequencing are limited, and the more time points included in the analysis, the better at revealing the transcriptome changes in glial cells as AD progresses. In the future, we hope to further explore the precise interactions between glial cells. This will allow us to more accurately understand the roles of other glial cells and their interaction in AD. In addition, we would perform single-cell sequencing at more time points to understand DAM in greater detail. We can track progress more precisely and understand the transition between the early and late stages in DAM.

In conclusion, we further characterized the DAM and found it to be time-point dependent. We confirmed that Trem2 plays a significant role in DAM activation. However, we also found some recent Trem2-dependent markers to be Trem2 independent here. Lastly, we showed the cross-talk between other glial cells. Using the insight provided by the data in this paper, new directions for future research were identified. Furthermore, incorporating new insights might allow us to identify one specific target or several targets for future drug development as well as potential off-target targets that require attention, thereby increasing the potential safety and accuracy of future drug development.

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Supplementary data

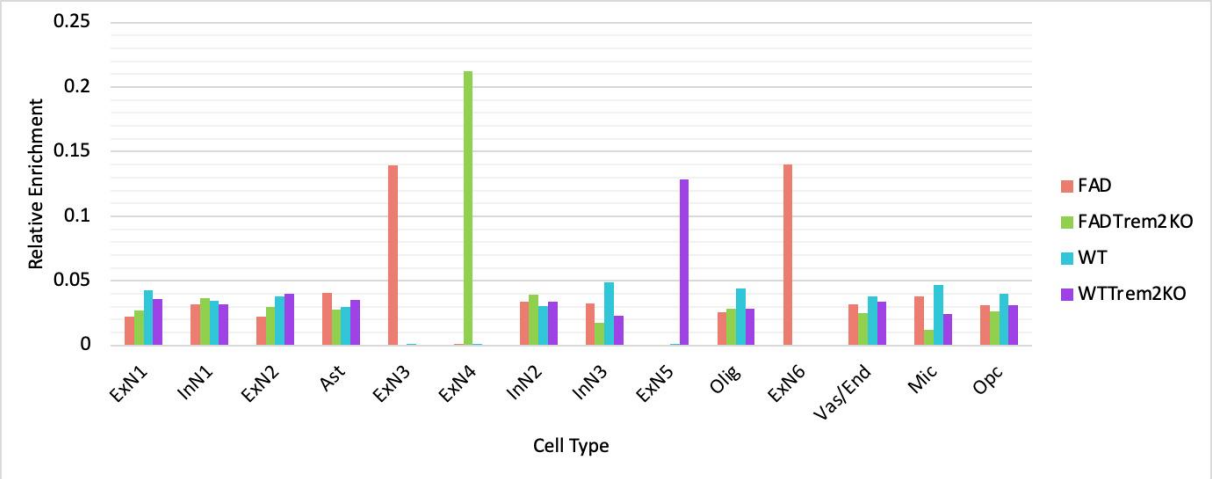
Differentially expressed genes in hippocampus, FDR<0.05

Age	high in FAD	high in WT	high in FAD, DAM	high in FAD, homo	high in WT, DAM	high in WT, homo
4	25	2	12	1	0	0
8	135	1	26	10	0	0
12	274	16	46	17	0	1
18	512	152	48	19	2	1

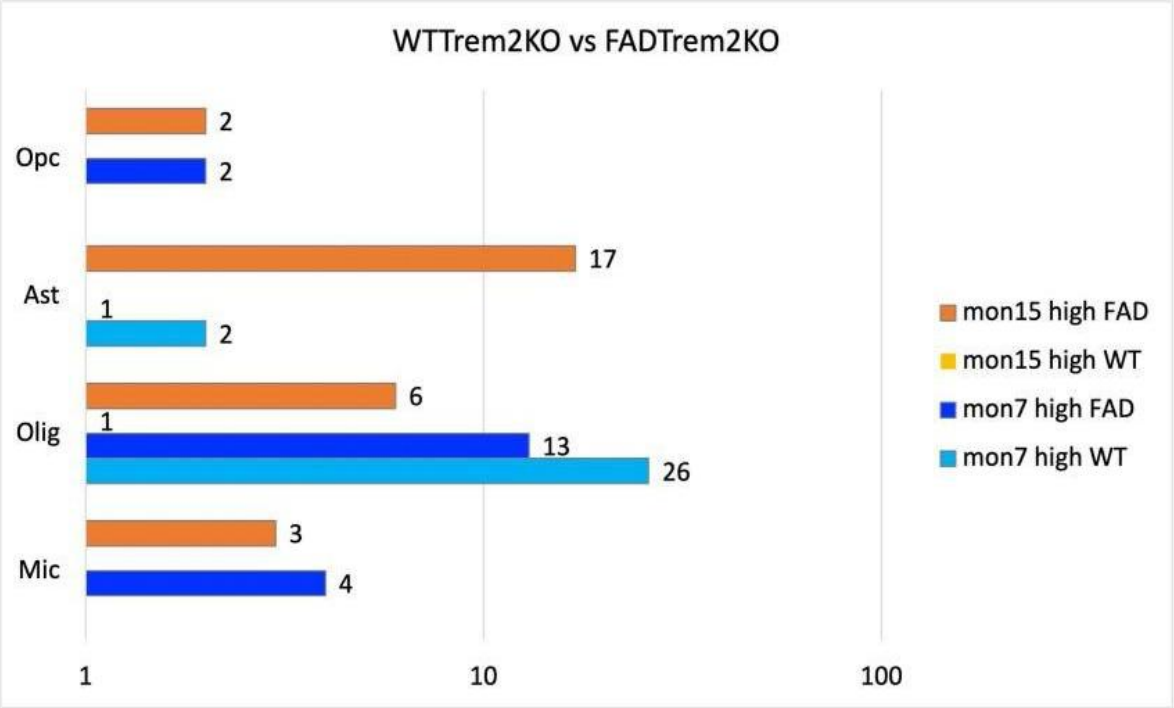
Differentially expressed genes in cortex, FDR<0.05

Age	high in FAD	high in WT	high in FAD, DAM	high in FAD, homo	high in WT, DAM	high in WT, homo
4	1	0	0	0	0	0
8	56	2	15	3	0	1
12	88	11	21	1	0	0
18	208	21	40	11	0	0

Supplementary Table 1



Supplementary Figure 1



Supplementary Figure 2