

Naturally-aged microglia exhibit phagocytic dysfunction accompanied by gene expression changes reflective of underlying neurologic disease

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Article

Keywords:

Posted Date: June 23rd, 2022

DOI: <https://doi.org/10.21203/rs.3.rs-1720794/v1>

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Abstract

Age-associated microglial dysfunction contributes to the accumulation of amyloid- β (Ab) plaques in Alzheimer's disease. Although several studies have shown age-related declines in the phagocytic capacity of myeloid cells, relatively few have examined phagocytosis of normally aged microglia. Furthermore, much of the existing data on aging microglial function have been generated in accelerated genetic models of Alzheimer's disease. Here we found that naturally aged microglia phagocytosed less Ab over time. To gain a better understanding of such dysfunction, we assessed differences in gene expression between young and old microglia that either did or did not phagocytose Ab. Young microglia had both phagocytic and neuronal maintenance signatures indicative of normal microglial responses, whereas, old microglia, regardless of phagocytic status, exhibit signs of broad dysfunction reflective of underlying neurologic disease states. We also found downregulation of many phagocytic receptors on old microglia, including TREM2, an Ab phagocytic receptor. TREM2 protein expression was diminished in old microglia and loss of TREM2⁺microglia was correlated with impaired Ab uptake, suggesting a mechanism for phagocytic dysfunction in old microglia. Combined, our work reveals that normally aged microglia have broad changes in gene expression, including defects in Ab phagocytosis that likely underlies the progression to neurologic disease.

Introduction

Alzheimer's disease is partially characterized by the accumulation of amyloid- β (Ab) plaques in the brain that impair cognitive function^{1,2}. Ab plaque accumulation increases with age and interestingly, correlates with known deficiencies in immune cell function. Although Ab is normally produced in the brain, its accumulation is prevented by the brain's resident phagocytic cell, the microglia³. In fact, phagocytic deficiencies in microglia are strongly linked to Alzheimer's disease progression^{4,5,6}. Thus, microglia are central regulators of Ab homeostasis, and in doing so, act as gatekeepers to Alzheimer's disease. However, although greater than 95% of cases are considered age-related sporadic Alzheimer's disease and the likelihood of developing Alzheimer's disease exponentially increases after the age of 65⁷, many of the studies linking phagocytic deficiencies in Alzheimer's disease have employed transgenic mouse models that overproduce Ab, overwhelm microglial phagocytic capacity, and artificially accelerate disease development⁸. The few studies that have examined microglial function in normally aged mice reported defects in phagocytic dysfunction, including phagocytosis of Ab as early as 12 months of age^{9,10}. However, as 12-month-old mice represent middle aged humans, the microglial phagocytosis of Ab in old mice (>16 months-old) remains unknown.

Here, we investigated the role normal aging plays on microglial phagocytic function. Consistent with reported deficiencies in phagocytic function of other myeloid cells^{11,12,13,14}, we found that both the frequency and per cell phagocytic capacity were reduced in aged microglia. Mechanistically, aged microglia had decreased expression of several phagocytic receptors including triggering receptor expressed on myeloid cells 2 (TREM2) which correlated with decreased ability to phagocytose Ab. Further,

bulk mRNAseq analysis revealed that young microglia displayed increase expression of genes important in phagocytosis, as well as neuronal maintenance whereas aged microglia, regardless of phagocytosis status, had increased expression of gene associated with proliferation.

Materials And Methods

Mice

Young (≤ 3 months) and aged (≥ 18 months) male and female C57Bl/6 mice were used for all experiments. Aged mice were obtained from the National Institute of Aging (NIA) colony located at Charles River Laboratories (Wilmington, MA) and young mice were bred in house or obtained from the NIA. Mice obtained from NIA were allowed to acclimate to our mouse facility for at least one week before experimentation. All animal procedures were performed in accordance with relevant guidelines and regulations, including the Animal Research: Reporting in Vivo Experiments (ARRIVE) guidelines and were reviewed and approved by the Institutional Animal Care and Use Committee at the Cincinnati Children's Hospital Research Foundation (IACUC 2019-0049).

Microglial Enrichment

The protocol for enrichment of immune cells was adapted from ¹⁵. Briefly, immediately after euthanasia by CO₂ inhalation, mice were perfused by inserting a 27G butterfly needle into the apex of the heart's left ventricle, incising the right atrium, and pumping ~30mL of cold sterile PBS through the mouse until the liver paled. After perfusion, the brains were harvested from the mice and manually dissociated through a 100 μ m cell strainer to create a single cell suspension. Next, the samples were further digested by incubating with 2U/mL of Liberase with low thermolysin concentration (Roche Diagnostics) for 1 hour. After the digestion incubation, the cell suspension was sieved through a 70 μ m cell strainer and rinsed thoroughly with HBSS containing 666U/ml DNase I (Roche Diagnostics). After pelleting cells, myelin and cellular debris was removed by resuspending in a 25% Percoll Plus (GE Healthcare) density gradient medium where immune cells pelleted after centrifugation (521 x g for 20 minutes).

A β 42 Peptide Phagocytosis Assay and Flow Cytometry

Amyloid Beta 1-42 peptide (A β 42) conjugated to HiLyte 488 (Anaspec, Fremont, CA) was incubated at 37°C for at least 12 hours to fibrilize prior to the phagocytosis assay. A β 42 was added to culture with either young or aged enriched microglia. Duration of culture is noted in each figure. After microglial enrichment and/or phagocytosis assay, samples were stained for flow cytometry using a fixable LiveDead viability stain (ThermoFisher Scientific) and surface stained with the following antibodies: anti-CD45 (clone: 104), -CD11b (clone: M1/70) (Biolegend), and -TREM2 (clone: 237920) (R&D Systems). A β 42 uptake was evidenced by the presence of HiLyte 488 in microglia (CD45 intermediate and CD11b⁺ cells) and was quantified via flow cytometry. Data was acquired on a FACSCanto flow cytometer from BD Biosciences and analyzed using FlowJo software (Ashland, OR) or an imaging flow cytometer (Amnis ImageStreamX Mark II, Luminex).

A β 42⁺ /- Microglia Flow Sorting, RNA extraction, and RNA sequencing

After microglial enrichment and phagocytosis of A β 42 peptide, brain cells from 4 young and 4 old mice were stained for flow sorting using fixable LiveDead viability stain (ThermoFisher Scientific) and CD45 and CD11b antibodies (Biolegend). Young and old microglia (live, CD45^{int}, CD11b⁺) were sorted into two groups: A β 42 positive and A β 42 negative microglia using a FACS Aria II Cell Sorter (BD Biosciences). Directional polyA RNA-seq was performed by the Genomics, Epigenomics and Sequencing Core at the University of Cincinnati using established protocols as previously described^{16,17}. Briefly, total RNA quality was assessed by Bioanalyzer (Agilent, Santa Clara, CA). Using good quality total RNA as input, polyA RNA was isolated using NEBNext Poly(A) mRNA Magnetic Isolation Module (New England BioLabs, Ipswich, MA). Enrichment of the polyA RNA using SMARTer Apollo automated NGS library prep system (Takara Bio USA, Mountain View, CA) and concentration of the polyA RNA to 6 ul via CentriVap micro IR Vacuum Concentrator (Labconco, Kansas City, MO) was performed prior to library preparation. Next, libraries were prepared using NEBNext Ultra II Directional RNA Library Prep kit (New England BioLabs). After verifying library QC and quantification via real-time qPCR (NEBNext Library Quant Kit, New England BioLabs), individually indexed libraries were proportionally pooled and sequenced using NextSeq 550 sequencer (Illumina, San Diego, CA) using sequencing setting of single read 1x85 bp. After sequencing, Fastq files were automatically generated by Illumina BaseSpace Sequence Hub for downstream data analysis.

Differential gene expression analysis

RNA-seq reads in FASTQ format were first subjected to quality control to assess the need for trimming of adapter sequences or bad quality segments. The programs used in these steps were FastQC v0.11.5¹⁸, Trim Galore! v0.4.2¹⁹, and cutadapt v1.9.1²⁰. The trimmed reads were aligned to the reference mouse genome version mm10 with the program STAR v2.5.2a²¹. Aligned reads were stripped of duplicate reads with picard tools v1.89²². Gene-level expression was assessed by counting features for each gene, as defined in the NCBI's RefSeq database²³. Read counting was done with the program feature Counts v1.6.2 from the Rsubread package²⁴. Raw counts were normalized as transcripts per million (TPM). Differential gene expressions between groups of samples were assessed with the R package DESeq2 v1.26.0²⁵. Plots were generated using the ggplot2²⁶ package and base graphics in R. Pathway analysis was performed by using ToppGene Suite²⁷ where differential expressed genes that had a p value of ≤ 0.1 and a $\log_2(\text{Fold Change}) \geq 0.5$ were used as the input.

Statistics

Statistical analysis was performed using GraphPad Prism. Data are presented as \pm standard error of mean (SEM). Significance was tested with Student's t test for differences between two groups. For line graphs, line of best fit equations were determined for each line and compared using simple linear regression. The p values of <0.05 were considered to be significant and are indicated with the following nomenclature: * $p < 0.05$

Results And Discussion

Loss of phagocytic capacity in microglia from aged mice

One of the main functions of microglia is their ability to recognize and phagocytose cellular debris including neurotoxic molecules such as A β , whose accumulation can impede cognitive function leading to Alzheimer's disease²⁸. To determine the effect of normal aging on microglial phagocytic function, we assessed phagocytic uptake of A β in primary microglia (identified as CD45^{int} and CD11b⁺, Suppl. Figure 1) from young and aged mice. Immune cells were isolated from the brains of young (≤ 3 months) and aged (≥ 18 months) C57Bl/6 mice, cultured with and without fibrilized fluorescently conjugated amyloid- β 1-42 (A β 42) for 1 hour, and phagocytosis was assessed by flow cytometry (Figure 1a). While the overall frequency of microglia was not altered in aged mice, the frequency of microglia that phagocytosed A β 42 was significantly reduced in aged mice compared to their younger counterparts (Figure 1b, c). As flow cytometry is unable to distinguish cell surface binding of A β 42 versus phagocytosis/internalization, we utilized imaging flow cytometry to confirm A β internalization by microglia (Suppl. Figure 2).

Next, to determine whether the defect in *in vitro* A β phagocytosis was observed at later timepoints, we assessed A β 42 uptake via flow cytometry at 1, 3, 6, and 12 hours after incubation. Although the percentage of A β 42⁺ young and old microglia steadily increased over time, the percentage of old A β 42⁺ microglia continued to trail that of young microglia (Figure 2a, b). Additionally, the amount of phagocytosed A β 42, as measured by the fluorescence intensity per cell (geometric mean of fluorescence, gMFI), in old microglia was lower than in their younger counterparts, reaching their phagocytic maximum by 12 hours compared to young microglia whose gMFI continued to increase (Figure 2c). These data show that aged mice have fewer microglia capable of A β 42 phagocytosis and, on a per cell basis, phagocytose less A β 42 than those from young mice.

RNAseq Analysis Reveals Pervasive Aged Microglial Dysfunction

Given that the frequency and capacity of aged microglia to phagocytose A β was reduced, we took an unbiased approach to understand gene expression changes in aged microglia that could contribute to their defective phagocytosis. To do this, we cultured aged and young microglia with fluorescent A β 42, FACs sorted microglia that did (A β 42⁺) and did not (A β 42⁻) phagocytose and we subjected them to bulk mRNAseq (Figure 3a). To determine the relationships between these sorted populations, we performed principal component analysis (PCA). PCA showed that phagocytosing (A β 42⁺) young and old microglia clustered together suggesting that they had very similar gene expression (Figure 3b, compare dark red circles to black circles). In contrast, young and old microglia that did not phagocytose (A β 42⁻) cluster distantly from those that did phagocytose (A β 42⁺). Further, aged microglia that did not phagocytose A β 42 clustered further away from all other subsets (Figure 3b, compare gray circles to all other circles). Together, these data show that age-associated changes in gene expression are associated with microglial phagocytic dysfunction.

To gain insight into the biology underlying defective phagocytosis with age, we generated lists of differentially expressed genes (DEGs) and performed pathway analysis. In those microglia that phagocytosed A β 42, there were 3402 genes that were upregulated in young compared to old microglia and 856 genes that were increased in old compared to young microglia (Table 1). Pathway analysis showed that young microglia had increased expression of genes involved in endocytosis, membrane trafficking genes, and proteolysis (Figure 3c). Notably, relative to old microglia, young microglia showed increased expression of RAB and ARF genes (Suppl. Figure 3a) which are known to be involved in endosomal trafficking, vesicle formation, and intracellular transport²⁹. Given that defective function of both of these families of small Ras GTPases have been implicated in neurodegenerative diseases³⁰, it is possible that their decreased expression could contribute to reduced microglial phagocytic capacity and contribute to disease in aged microglia.

Following phagocytosis, ingested material is broken down into reusable substrates. Interestingly, young microglia had increased expression of genes associated with proteasomal subunits (*Psmc1*, *Psmc3*, *Psmc11*, *Psmc12*, and *Psmc14*) as well as immunoproteasome subunits (*Psmc6* and *Psmc7*) (Suppl. Figure 3b). This data is consistent with prior work suggesting decreased proteasomal processing in aged, senescent cells³¹. Further, young microglia that phagocytosed A β 42 had increased expression of several cytokine and cytokine receptor genes relative to old microglia. Increased mRNA levels of *Il1a*, *Il18*, *Il12b*, *Ifng*, and *Csf1* in the young microglia indicated a robust pro-inflammatory profile (Figure 3c, Suppl. Figure 3c). Furthermore, cytokine receptors such as *Il1rl2*, *Il12rb2*, *Il6ra*, *Il10ra*, and *Csf1r* (Suppl. Figure 3c) and genes involved in cytokine signaling including, TRAF, SMAD, and NFKB genes (Suppl. Figure 3d), were also highly expressed likely enabling these young microglia to respond to multiple cytokines. Overall, the increase in immune cell activation genes show that young phagocytosing microglia are capable and prepared to respond to immunological signals and targets. We acknowledge that upregulation of pro-inflammatory markers in the young microglial compartment compared to old microglia is not in agreement with previous studies that reported increased inflammatory signatures in other aging cells such as macrophages and dendritic cells^{32, 33}. However, a recent single cell RNAseq study showed that only a small proportion of aging microglia have an inflammatory signature³⁴, suggesting that microglia are not the primary source of inflammaging in the brain. Although we note that our bulk mRNAseq approach would not reveal subtle trends present only in subpopulations and can only unveil the pathways present in most microglia.

In contrast to the young phagocytosing microglia, pathway analysis of young microglia that did not phagocytose A β 42 revealed increased expression of genes associated with extracellular matrix and synaptic interaction (Table 1). Young microglia had significantly increased expression of several matrix metalloproteinases (*Mmp3*, *Mmp11*, *Mmp12*, *Mmp15*, *Mmp16*) and ADAM metalloproteinases (*Adamts4*, *Adamts3*, *Adamts2*, *Adam23*, *Adam7*) (Figure 3d, Suppl. Figure 3e). Recent studies have reported that upregulation of ECM correlates with remodeling of neuron synapses^{35, 36}, thus suggesting that these young non-phagocytosing microglia may play a very different role than their phagocytosing counterparts. Notably, microglia are important for remodeling and maintenance of neuronal synapses and increased

expression of these metallopeptidases is likely critical for this process^{35, 37, 38}. Neuronal-type pathways, such as “neuronal system”, “neuroactive ligand-receptor interactions”, and “protein-protein interactions at synapses”, were also notably increased in these young non-phagocytosing microglia (Figure 3d), suggesting that non-phagocytosing microglia are programmed more for neuronal remodeling rather than immune function. This bifurcated functionality of young microglia is in agreement with the concept of heterogeneity of the microglial compartment, an expanding area of interest in neuronal research^{39, 40}. These data suggest that there are microglia “pre-primed” to respond to damage signals like A β that can be activated very quickly after recognition of these signals, while other microglial populations are mainly involved in neuronal remodeling and maintenance.

Pathway analysis of old phagocytosing microglia indicates a very different cellular state from that of their young counterparts. Cell cycling and associated pathways dominated as the top pathways in these cells (Figure 3c). Cyclins and cyclin-dependent genes (*Ccnb1*, *Ccnb2*, *Cdk1*, *Cdkn2c*), cell division cycle genes (*Cdca5*, *Cdc20*, *Cdc25b*, *Cdca8*) and nucleosome assembly genes (*Cenpn*, *Cenpk*, *Cenpl*) were notably upregulated in the old phagocytosing microglia. Interestingly, increased proliferative capacity also appears to be a defining factor in old microglia that did not phagocytose (Figure 3d, Suppl. Figure 3f and 3g), along with upregulation of DNA repair genes. Such unregulated cell cycling could be damaging to the microglia. Indeed, increased microglial proliferation is an early characteristic of disease-associated microglia (DAMs)⁴¹, which have been reported in multiple mouse models of neurodegenerative diseases such as Alzheimer’s disease⁴², prion disease⁴³, and ischemic stroke⁴⁴. However, despite this increase in proliferation, we did not see increases in overall percentage of microglia in aged mice. This may be due to proliferative stress, indicated by upregulation of DNA repair pathways and PLK1 signaling, in aged microglia. Thus, aged cycling microglia are likely to be prone to cell death that prevents increased microglia numbers with age. Additionally, such increased cell cycling may place intense energetic demands on the aged cells, a concept supported by the fact we also found upregulated expression of metabolic-related genes implicated in the citric acid cycle (TCA) and the electron transport chain (ETC) (Figure 3d).

Beyond a proliferative phenotype, pathway analysis of old microglia that did not phagocytose also revealed pathways associated with neurodegenerative diseases, such as *App*, *Adam17*, *Psen1*, and *Psen2*^{45, 46} (Suppl. Figure 3h). Additionally, old microglia that did phagocytose had an upregulation of the amyloid fiber formation pathway (Figure 3c) further hinting those old microglia, regardless of their phagocytic status, exhibit tell-tale signs of microglial dysfunction and disease state.

Decreased TREM2 expression in Aged Microglia Correlate with Phagocytic Uptake

Given the dysfunctional phagocytosis in aged microglia, we next examined the differences in gene expression of over 20 receptors potentially involved in phagocytosis. Old microglia that did not phagocytose A β 42 had low expression of two-thirds of these genes. Expression of these genes encoding these receptors was decreased compared to not only their younger non-phagocytosing counterparts but

also to old microglia that did phagocytose (Figure 4a), suggesting that decreases in phagocytic capacity may be due to a population of old microglia losing their ability to recognize phagocytic material. Interestingly, we found that mRNA expression of one phagocytic receptor, TREM2, was particularly low. Studies have shown that TREM2 directly binds A β ⁴⁷, is a vital component of A β clearance⁴⁸, and has been linked to Alzheimer's disease in both Alzheimer's patients^{49, 50} and in mouse models^{51, 52}. Many studies concluded that TREM2 expression is positively correlated with uptake of A β ^{53, 54} and loss of TREM2 function leads to A β accumulation⁵⁵, although a recent study found that TREM2 deficiency increased phagocytosis in an Alzheimer's disease mouse model⁵⁶. Overall, the literature suggests that normal age-related declines in TREM2 expression may contribute to defective phagocytosis in aged microglia.

To confirm these differences in TREM2 mRNA levels, we next assessed protein levels of TREM2 on young and old microglia by flow cytometry. Aged mice had an overall decrease in the frequency of TREM2⁺ microglia compared to their younger counterparts (Figure 4b), consistent with prior studies that showed decreased *Trem2* mRNA expression in aged microglia⁵⁷. We next asked whether TREM2⁺ cells were the predominant microglial population involved in A β phagocytosis. In young mice, nearly 75% of A β 42⁺ microglia expressed TREM2, while in aged mice, only 50% of aged A β 42⁺ microglia were TREM2⁺ (Figure 4c, 4d). However, the frequency of TREM2⁺ microglia that were A β 42⁺ was similar in young and aged mice (Figure 4e), suggesting that old TREM2⁺ microglia are still capable of taking up A β 42, but that other receptors likely contribute to A β 42 phagocytosis in aged microglia. Combined, these data suggest that the decreased proportion of TREM2⁺ old microglia contributes to the overall decreased ability to phagocytose A β peptide, which might contribute to A β accrual during late-onset Alzheimer's disease.

In summary, our data show that microglia from normally aged mice exhibit many tell-tale signs of dysfunction, including deficits in phagocytosis of A β peptide and substantially altered expression of genes involved in cell cycling, synaptic pruning and neuronal homeostasis. Importantly, much of the existing data on microglial function in aging have been generated in accelerated genetic models of Alzheimer's disease^{58, 59, 60} which may not reflect normal progressive inflammatory environment that develops with age. Our study highlights the importance of further research on age-related dysfunctions of microglia and how they contribute to driving age-associated sporadic disease.

Declarations

Acknowledgements

This research was supported by the National Institute of Aging (NIA grant: R01AG053498). All flow cytometric data were acquired using equipment maintained by the Research Flow Cytometry Core in the Division of Rheumatology at Cincinnati Children's Hospital Medical Center which is supported in part by NIH AR070549.

Author Contributions

A.L.T. contributed to experimental design, performed experiments, analyzed data, and drafted and revised the manuscript. M.A.L. performed experiments. E.M.J., C.A.C., and D.A.H. conceived the study, contributed to experimental design, reviewed and edited the manuscript, and supervised A.L.T. All authors read and approved the final version of the manuscript.

Competing Interests

The author(s) declare no competing interests.

Data Availability

The RNAseq dataset generated and analyzed in this current study is available in the Gene Expression Omnibus (GEO) repository (Accession # GSE205803).

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Tables

Table 1: Number of genes upregulated in young and old microglia that did and did not phagocytose A β 42.

	Up in Young	Up in Old
A β 42 ⁺	3403	857
A β 42 ⁻	5245	5539

Figures

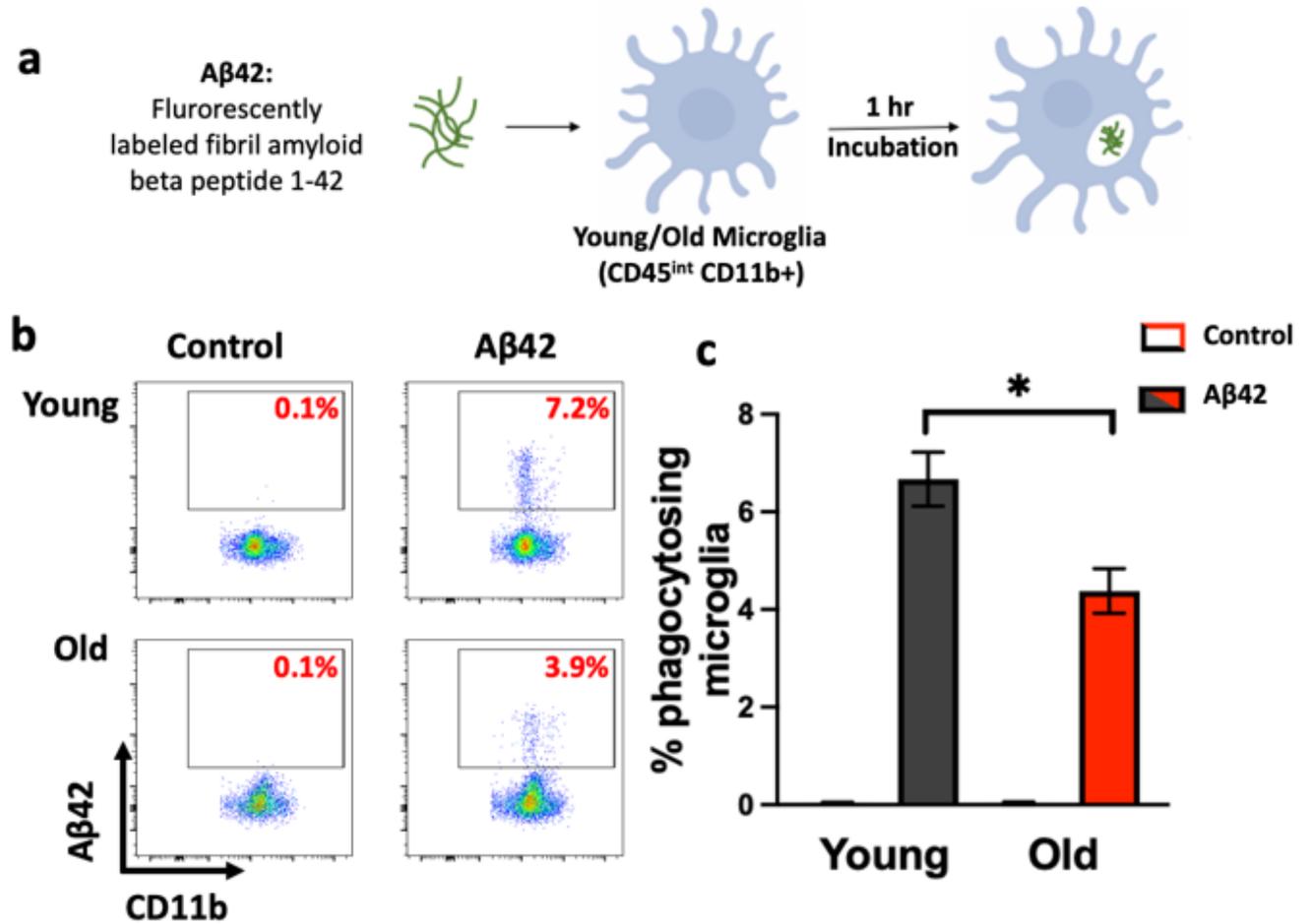


Figure 1: Aged microglia have reduced phagocytic capacity. Brains from female young and old mice (n=3) were processed and live CD45 intermediate CD11b⁺ cells (microglia) were assessed for phagocytic capacity. **a)** Cells were incubated with 0.5 μ M of fluorescent fibril A β 42 or medium (control) for 1 hour and phagocytosis was assessed by flow cytometry. **b-c)** Representative flow plots are shown and mean \pm s.e.m % of microglia showing phagocytosis of fluorescent A β 42 are graphed * $p \leq 0.05$, Student's *t* Test

Figure 1

See image above for figure legend

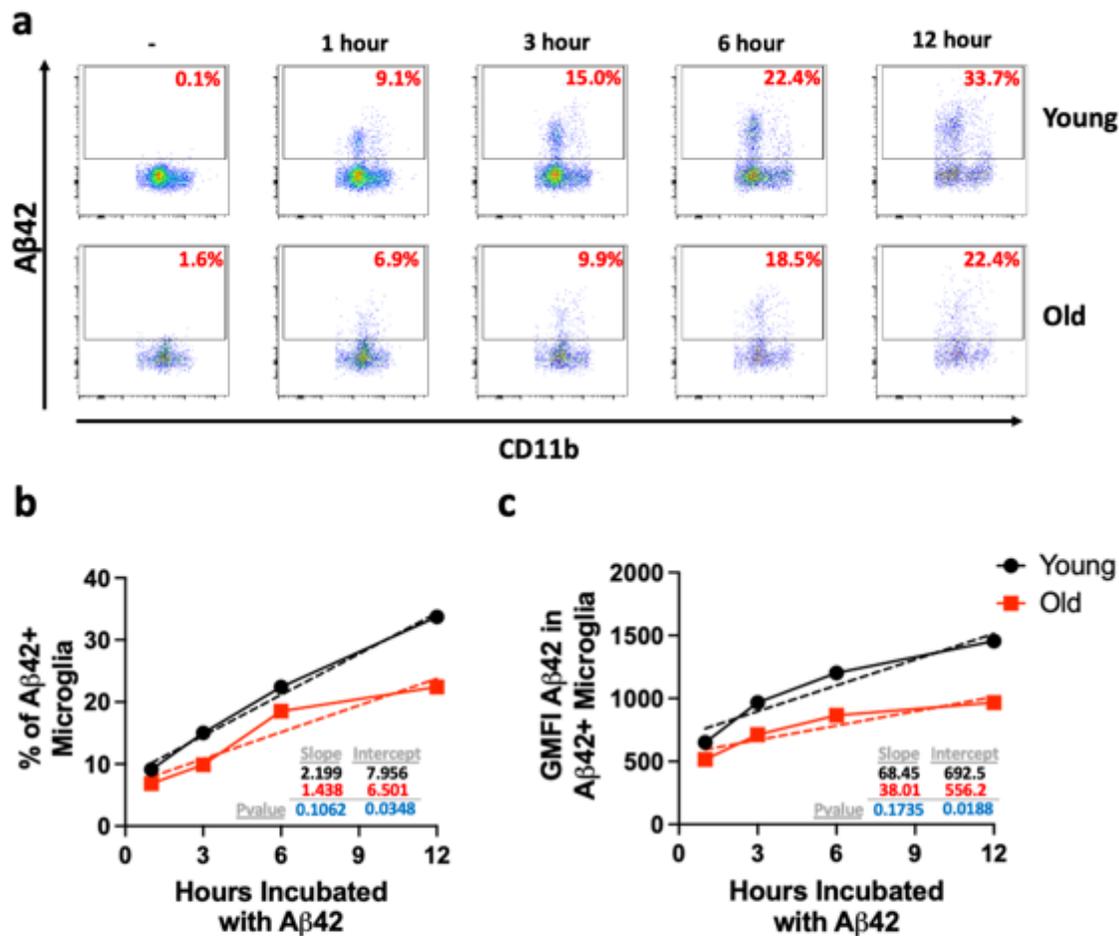


Figure 2: Aged microglia have less uptake capacity of Aβ overtime than their younger counterparts. Microglia from 3 young or old mice were isolated, combined with their age-matched counterparts, and divided into 5 time points where they were incubated with 2.5μM of Aβ42 for 1, 3, 6, and 12 hours. Microglia were analyzed via flow cytometry to measure phagocytosis. **a)** Representative flow plots showing uptake of Aβ42 by microglia over time. **b)** % of microglia that are Aβ42 positive over time. Dashed lines indicate line of best fit. **c)** Geometric mean of Aβ42 in Aβ42⁺ microglia. Dashed lines indicate line of best fit. Simple linear regression was used to determine differences between the lines of best fit.

Figure 2

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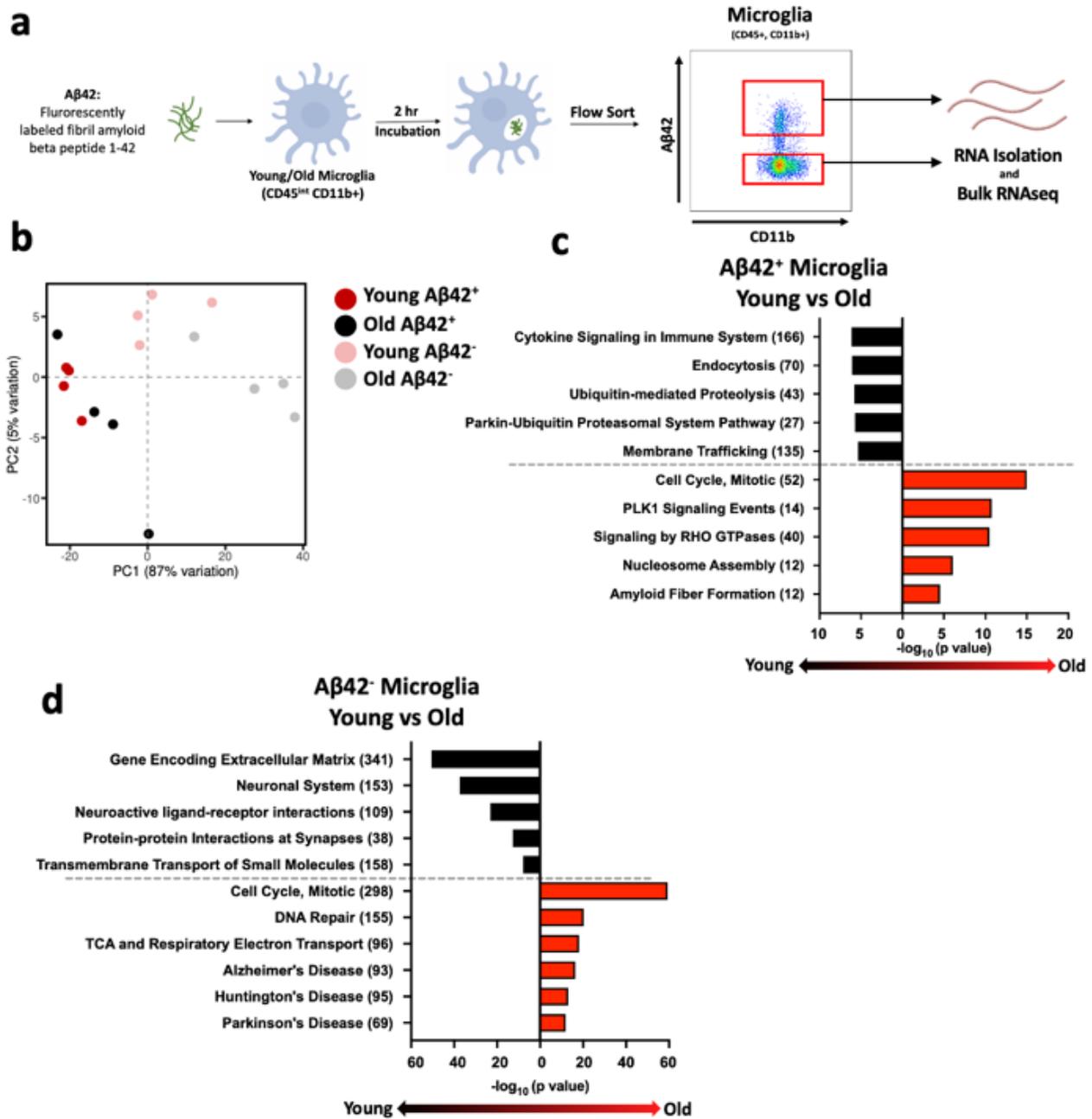


Figure 3: Pathway analysis of young and old microglia that either did or did not take up peptide showed deficiencies of old microglia. Microglia from three young and old mice ($n=4/\text{group}$) were combined and incubated with $2.5\mu\text{M}$ of $\text{A}\beta_{42}$ for 2 hours and then were FACSsorted based on $\text{A}\beta_{42}$ uptake. Sorted microglia's RNA was isolated and used to run bulk RNAseq analysis. **a)** Schematic of phagocytosis assay and flow sorting. **b)** Principal component analysis plot of the four different microglia groups (Young $\text{A}\beta_{42}^+$, Young $\text{A}\beta_{42}^-$, Old $\text{A}\beta_{42}^+$, Old $\text{A}\beta_{42}^-$) with 4 replicates each. Pathway analysis of DEGs in both the young and old **c)** $\text{A}\beta_{42}^+$ and **d)** $\text{A}\beta_{42}^-$ microglia graphed based on their p value. The number of DEGs in each pathway are indicated in parentheses.

Figure 3

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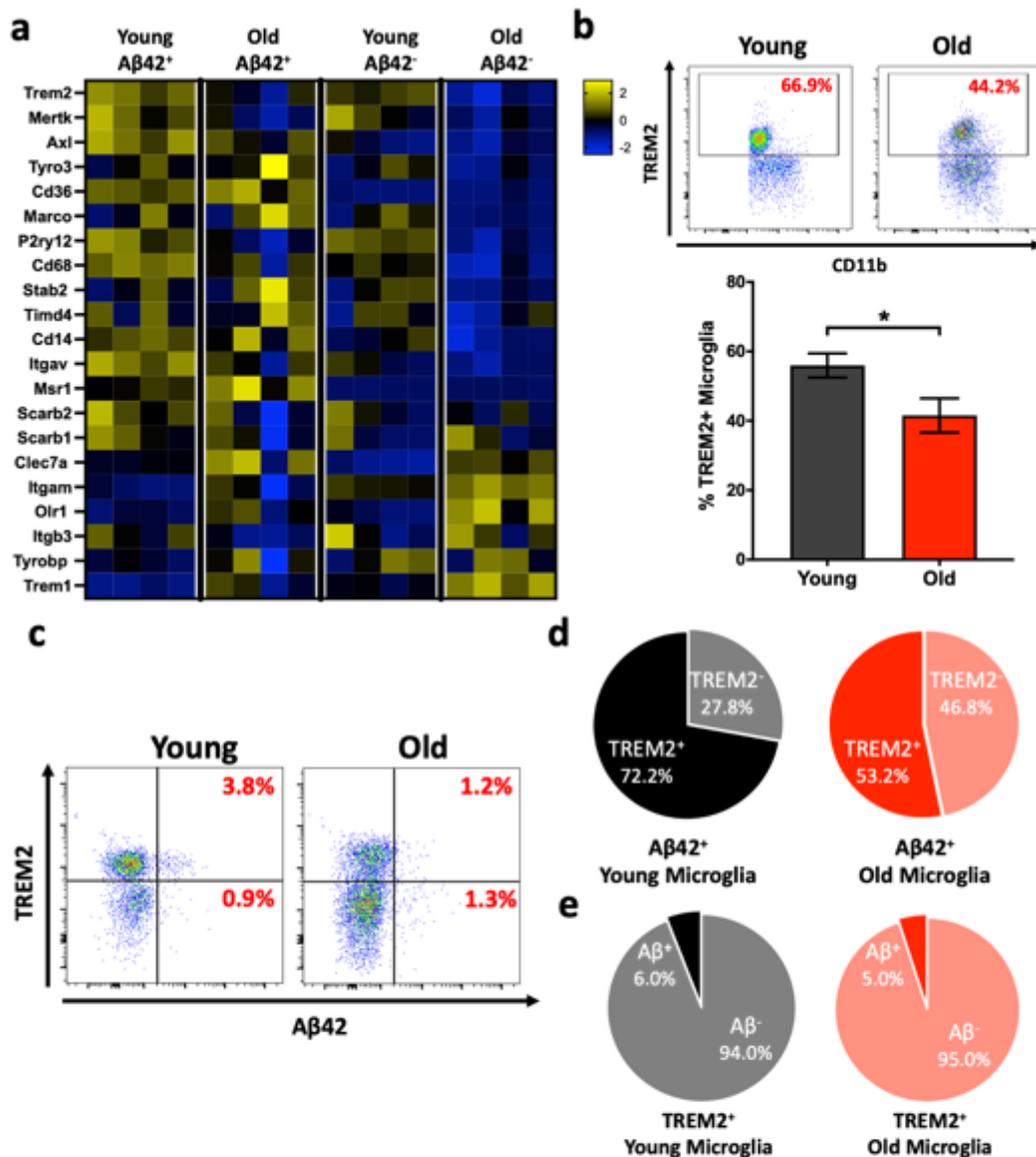


Figure 4: Expression of TREM2, an Aβ42 receptor, is decreased in aging microglia. a) Heatmap representation of phagocytic receptor gene expression levels for Young Aβ42⁺, Young Aβ42⁻, Old Aβ42⁺, and Old Aβ42⁻. The scale represents the row Z-score from 2 (highest expression) to -2 (lowest expression). Young and aged microglia were incubated with 0.5μM Aβ42 for 1 hour and stained for microglial markers and TREM2. b) Representative flow plots (n=3/group) of young and aged microglia TREM2 expression and graph of % of young and old microglia that are TREM2⁺. c) Representative flow plots (n=3/group) of young and aged microglia TREM2 expression and Aβ42 uptake. d) Graphs showing the average percentage of TREM2 expression in young (black) and old (red) Aβ42⁺ microglia and e) Average percentage of Aβ fluorescence in young (black) and old (red) TREM2⁺ microglia. *p ≤ 0.05, mean ± s.e.m, Student's t Test

Figure 4

See image above for figure legend

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