Age-dependent microglial disease phenotype results in functional decline in gut macrophages


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Muscularis Macrophages

Homeostatic State
- Tissue protective
- Microglial genes
- Phagocytic activity
- Intracellular α–Synuclein

Geriatric State
- Pro-inflammatory
- DAM genes
- Phagocytic activity
- Intracellular α–Synuclein

aging, neurodegeneration
Age-dependent microglial disease phenotype results in functional decline in gut macrophages

Short Title: Microglial genes in ENS macrophages and aging

Estelle Spear Bishop\(^a\), Hong Namkoong\(^a\), Laure Aurelian\(^b,c\), Madison McCarthy\(^d\), Pratima Nallagatla\(^e\), Wenyu Zhou\(^e,f\), Leila Neshatian\(^a\), Brooke Gurland\(^d\), Aida Habtezion\(^a\)* and Laren Becker\(^a,1\)*

\(^a\)Department of Medicine, Division of Gastroenterology and Hepatology, Stanford University; Stanford, California 94305; \(^b\)Stanford University School of Medicine, Stanford, CA 94305; \(^c\)Department of Pharmacology, University of Maryland School of Medicine; Baltimore, Maryland 21201; \(^d\)Department of Surgery, Stanford University; Stanford, California 94305; \(^e\)Stanford Center for Genomics and Personalized Medicine, Stanford University; Stanford, California 94305; \(^f\)Department of Genetics, Stanford University; Stanford, California 94305

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Abbreviations: DAM (disease-associated microglia), ENS (enteric nervous system), GS (geriatric state), HS (homeostatic state), LpM (lamina propria macrophage), MM (muscularis macrophage)

Bold denotes dual first authorships.

\(^1\)To whom correspondence should be addressed: lsbecker@stanford.edu

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ABSTRACT

BACKGROUND & AIMS: Muscularis macrophages (MMs) are tissue resident macrophages in the gut muscularis externa that provide a supportive role to the enteric nervous system (ENS). We have previously shown that age-dependent MM alterations drive low-grade ENS inflammation, resulting in neuronal loss and disruption of gut motility. The current studies were designed to identify the MM genetic signature involved in these changes, with particular emphasis on comparison to genes in microglia, the central nervous system macrophage population involved in age-dependent cognitive decline. METHODS: Young (3 months) and old (16-24 months) C57BL/6 mice and human tissue were studied. Immune cells from mouse small intestine, colon and spinal cord, and human colon were dissociated, immunophenotyped by flow cytometry, and examined for gene expression by single cell RNAseq and quantitative RT-PCR. Phagocytosis was assessed by in vivo injections of pHrodo beads. Macrophage counts were performed by immunostaining of muscularis whole mounts. RESULTS: MMs from young and old mice express homeostatic microglial genes, including Gpr34, C1qc, Trem2 and P2ry12. An MM subpopulation that becomes more abundant with age, assumes a geriatric state (GS) phenotype characterized by increased expression of disease-associated microglia (DAM) genes including CD9, CLEC7A, Itgax (CD11c), Bhlhe40, Lgals3, IL-1β, and Trem2, and diminished phagocytic activity. Acquisition of the GS phenotype is associated with clearance of α-synuclein (α-SYN) aggregates. Human MMs demonstrate a similar age-dependent acquisition of the GS
phenotype associated with intracellular α-SYN accumulation. **CONCLUSION:** MMs demonstrate age-dependent genetic changes that mirror the microglial DAM phenotype and result in functional decline.

**Keywords:** Enteric nervous system, muscularis macrophages, microglia, aging

**INTRODUCTION**

Muscularis macrophages (MMs) are tissue-resident macrophages (TRMs) located in the gut muscularis externa in close proximity to enteric neurons that provide a supportive role to the enteric nervous system (ENS). MMs sense cues in the ENS microenvironment including those from neurons and the gut microbiota, and relay the information to neighboring enteric neurons and smooth muscle cells\(^1\)\(^{-8}\). Unlike macrophages from the mucosal gut layer [termed lamina propria macrophages (LpMs)] that preferentially express pro-inflammatory associated genes (M1 phenotype), MMs display an anti-inflammatory M2 phenotype\(^3\). Alterations in MM phenotype have been implicated in gastrointestinal (GI) motility disorders, including gastroparesis, post-infectious GI dysmotility and post-operative ileus\(^6\)\(^,\)\(^8\)\(^{-13}\), and experimental MM depletion causes apoptosis of enteric neurons and disruption in gut motility\(^2\)\(^,\)\(^14\). We have previously shown that aging causes a shift in MM phenotype from anti-inflammatory to pro-inflammatory resulting in neuroinflammation that is associated with apoptosis of enteric neurons and disruption of gut motility\(^15\). However, whether these age-dependent functional alterations affect all MMs uniformly or just a subset, and the mechanism behind such changes remains poorly understood.
TRMs associated with peripheral nerves share common functions and genetic signatures with microglia, the macrophage population in the central nervous system (CNS)\textsuperscript{14,16-19}. The present studies were stimulated by the finding that microglia play a central role in age-related cognitive changes\textsuperscript{20}. Indeed, a microglial subpopulation in aged animals is characterized by diminished expression of genes involved in normal homeostatic cell functions and increased expression of disease-associated microglia (DAM) genes that are involved in pattern recognition, phagocytosis and leukocyte adhesion\textsuperscript{21-25}. We report that murine and human MMs express a microglial gene profile that changes with aging. MMs from aged animals express geriatric state (GS) genes that are similar to the microglial DAM genes. GS genes in both mouse and human MMs are induced by the intracellular accumulation of $\alpha$-synuclein ($\alpha$-SYN).

**METHODS**

Further details are provided in Supplemental Materials.

**Animals**

Specific pathogen free (SPF) C57BL/6 mice (3, 10 and 16-29 months old) were acquired from the NIA Aged Rodent Colony. All mice were housed in temperature and humidity controlled rooms with a 12 h light:dark cycle and maintained on *ad libitum* standard chow and water. Animal handling and procedures followed the National Institutes of Health Statement of Compliance with Standards for Humane Care and Use of Laboratory Animals and were approved by the Stanford University Institutional Animal Care and Use Committees.

**Human Samples**

All colon resection samples were obtained under an IRB-approved study at Stanford Hospital (IRB-55435, IRB-11977). Full thickness colon specimens were obtained from patients
undergoing colorectal surgery for indications including cancer, rectal prolapse, diverticular disease, constipation and volvulus (Table 1). For rectal prolapse cases, the most proximal part of the resected bowel was taken. For cancer cases, normal tissue margins, confirmed by histologic examination by a trained pathologist, were included in the study. Tissue was placed in freezing media (RPMI media with 80% FBS and 10% DMSO) or embedded in OCT (after fixation in 4% PFA and incubation in 30% sucrose) and stored at -80°C until further processing.

**Antibodies and Reagents**

Antibodies used in flow cytometry and immunofluorescent staining are listed in Supplemental Table 1. Media and reagents are listed in Supplemental Table 2.

**Tissue and Cell Preparation**

Mice were euthanized and laparotomy was performed. Small intestine (SI) and colon was removed, lavaged, and the muscularis externa was dissected and fixed in 4% PFA for whole mount preparations or enzymatically digested with collagenase and dispase, as previously described\(^\text{15}\). Following the removal of the muscularis externa the remaining lamina propria tissue was incubated with stirring (20 min; 37°C) in Hank’s Buffered Saline Solution (HBSS) with 2% bovine calf serum (BCS) and 2mM EDTA to deplete epithelial cells. After washing in PBS, tissue was enzymatically digested in collagenase and dispase, mechanically dissociated by gentle trituration and filtered through a 100 μm nylon mesh cell strainer for single cell preparation. For spinal cord cell isolation, the vertebral column was removed and a 20G needle containing 10mL of 1X PBS was inserted into the lumbar vertebral foramen for ejection of the intact spinal cord by irrigation. The spinal cord was sectioned into small pieces, and enzymatically digested with collagenase and dispase, mechanically dissociated by gentle
trituration, and filtered through a 100 μm nylon mesh cell strainer. Mononuclear cells were enriched (and myelin debris removed) by centrifugation in a percoll gradient.26

For human tissue, following dissection of muscle and mucosa layers, mucosal tissue was incubated with stirring (20 min x 2; 37°C) in HBSS with 2% bovine calf serum (BCS) and 2mM EDTA to deplete epithelial cells. After washing in PBS, muscularis and lamina propria were minced and enzymatically digested [30 minutes, 37°C (lamina propria); 45 minutes, 37°C (muscle) with spinning 400 rpm] in RPMI digestion buffer containing 5% BCA, 10mM HEPES, 0.25 mg/ml Liberase TL (Roche, Switzerland) and 20 U/ml DNase I. Cells were filtered through a 100 μm nylon mesh cell strainer for single cell preparation.

**Single Cell Transcriptomics**

CD45+ sorted cells were subjected to droplet-based 3’ end massively parallel single-cell RNA sequencing (scRNA-seq) using Chromium Single Cell 3’ version 3 library kits (10x Genomics), as per manufacturer’s instructions. The libraries were sequenced using Illumina HiSeq 4000 Sequencing System, with three pooled libraries on one lane. Sample demultiplexing, barcode processing, single-cell counting and reference genome mapping (refdata-cellranger-mm10-3.0.0) were performed using the Cell Ranger Single-Cell Software Suite (10x Genomics) according to the manual. The average total reads sequenced per sample were 110,059,643. An average of 87.6% of the reads were mapped to reference genome, which results in an average of 2,517 genes/cell sequenced in 488 cells/sample (Supplemental Table 3). Young and old samples were normalized to present the same effective sequencing depth by using the Cell Ranger aggr function. The dimensionality reduction by principal components analysis, the graph-based clustering using K-means algorithm, and t-SNE visualization were performed using
Cell Ranger. The cluster heat map analysis was performed using “NormalizeData” and “AverageExpression” functions in Seurat 3.0.

Flow Cytometry and Immunofluorescent Staining

Muscularis externa was fixed in 4% PFA for immunofluorescent staining or enzymatically digested with collagenase and dispase for flow cytometry\(^4,15\). For flow cytometry, dissociated cells were treated with 5% rat serum and mouse anti-CD16/CD32 antibody in FACS buffer (HBSS containing 2% BCS) for 20 minutes at 4\(^\circ\)C for mouse cells or 2% mouse serum and Fc-Receptor blocking solution for 15 minutes at room temperature for human cells. Cells were incubated with primary antibodies (Supplemental Table 1) or FMO controls (30 minutes at 4\(^\circ\)C). DAPI was used for live/dead cell determination. For intracellular \(\alpha\)-SYN staining, cells were first treated (10 min, 4\(^\circ\)C) with Zombie Aqua (Biolegend, San Diego, CA) for cell live/dead determination, followed by blocking and subsequent incubation (20 min, 4\(^\circ\)C) with antibodies for cell surface immune markers (Supplemental Table 1). Cells were subsequently fixed and permeabilized using the eBioscience Transcription Factor Staining Buffer Kit (as per manufacturer’s protocol) and stained with AlexaFluor488-conjugated \(\alpha\)-SYN antibody. After washing with FACS buffer, cells were passed through a 40 \(\mu\)m nylon mesh cell strainer prior to FACS analysis (BD LSRII, BD Bioscience, San Jose, CA) or cell sorting (BD Aria) into DMEM containing 10% FBS (for single cell transcriptomics) or Trizol (for qPCR). Analysis was performed using FlowJo Software (Tree Star Inc, Ashland, OR) and results are expressed as signal intensity calculated by mean fluorescence intensity (MFI). For immunofluorescence, mid-small intestine muscularis tissue (corresponding to jejunum) was permeabilized, blocked, and incubated with primary antibodies (2 days, 4\(^\circ\) C) followed (overnight, 4\(^\circ\) C) by fluorescently labeled secondary antibodies (Supplemental Table
1), and imaged with a Nikon C1 cooled CCD camera confocal microscope as previously described\textsuperscript{15}.

**RNA Isolation and Quantitative Real-time PCR**

RNA isolation and quantitative RT-PCR (qPCR) were performed as previously described\textsuperscript{4,15} using Taqman probes and primers listed in Supplemental Table 4. Relative expression between groups was analysed using the Pfaffl method\textsuperscript{27}.

**Micro-injections of pHrodo beads**

To evaluate the phagocytosis ability of macrophages, muscularis micro-injection was performed in young (5-8 week, n=8) or old (22-24 week, n=11) male mouse colons using pHrodo™ Green Zymosan Bioparticles™ Conjugate (Invitrogen). Mice were anesthetized with isoflurane and a laparotomy was performed. pHrodo Bioparticles (1 mg/ml) were injected into 5 sites (5 \(\mu\)L/site) of the muscularis externa between the proximal and middle colon using a 10 \(\mu\)L Gastight Syringe (Hamilton, 7653-01) equipped with a 34-gauge small hub RN needle (Hamilton, 207434-10). The abdomen was sutured and mice were sacrificed after 1 hour. The colon was removed, lavaged, and the muscularis externa was dissected and enzymatically digested in 3mL of collagenase and dispase during incubation in a 37°C water bath. After washing in PBS, tissue was enzymatically digested in collagenase and dispase, mechanically dissociated by gentle trituration, and filtered through a 100 \(\mu\)m nylon mesh cell strainer for single cell preparation.

**Human Immunofluorescent Staining**

Full thickness human colon samples approximately 1cm x 0.5cm in length were fixed in 4% PFA and then cryo-preserved by immersion in 30% sucrose. Tissue was embedded in Tissue Tek OCT compound and frozen at -80°C. Frozen tissue was sectioned at a thickness of 10 \(\mu\)M on a Leica
Cryostat (CM 1950), mounted onto coated slides, and stored at -80°C until staining. On the day of staining, slides were thawed and air-dried and tissue was circled with a hydrophobic pap pen. Following rehydration in PBS, tissue was permeabilized in 2% Triton-x 100 with 1% goat serum for 15 minutes, followed by blocking in 10% goat serum for 1 hour. Samples were incubated overnight in a humidified chamber at 4°C with primary antibodies followed by a room temperature (1 hour) incubation with secondary antibodies (Supplemental Table 1). Tissue was cover slipped using DAPI mounting medium (Vectashield H-1200). Slides were imaged on a Keyence BZ-X800 microscope at 40x magnification. Z-stack images were acquired using the optical sectioning function at a stack height of 0.6 µM (BZ X-800 Viewer), and one representative plane was selected by implementing full focus (BZ X-800 Analyzer).

Quantification and Statistical Analyses

For α-SYN quantification, the number of cells co-staining with antibodies to MHCII (MMs) and α-SYN were counted in 10 randomly selected fields containing enteric ganglia using a 60X objective. A minimum of 8 fields and 30 MMs (range 30-106, average 63) were analyzed per animal. Data are expressed as mean ± SEM and analyzed using t-test, one-way analysis of variance with Bonferroni’s multiple comparisons test, two-way analysis of variance with two-stage linear step-up procedure, simple linear regression, and Pearson correlation as detailed in the figure legends. Statistical analysis was performed with GraphPad Prism 9 (GraphPad Software Inc, La Jolla CA) and significance was established at p <0.05. Differential analysis of scRNA-seq data used Seurat 3.0 with “NormalizeData” and “FindMarkers” functions. The data from old and young mice were compared using the Wilcoxon ranked-sum method, and p <0.05
was used for significance after multiple hypotheses correction by the Benjamini & Hochberg method 29.

RESULTS

MMs share a genetic signature with microglia and peripheral nerve-associated macrophages

CD45+ cells from small intestine (SI) muscularis externa tissue of young (3 months, n=2) and old (24 months, n=3) male C57BL/6 mice were isolated by flow cytometry and examined for gene expression by single-cell RNA sequencing (scRNA-seq) using drop seq methods (Figure 1A). Unbiased t-SNE plot analysis of 1,705 cells by k-means clustering identified 5 cell clusters in both young and old mice (Figure 1B, C). These clusters were assigned as MMs (Cd163, Cd14; cluster 1), T cells (Cd3, Thy1, T cell receptor beta (Tcrb); cluster 2), immature B cells (Cd19, Cd79a; cluster 3), and mature B cells (Ighg2b, Ighm, Jchain; cluster 4) based on expression of known marker genes (Figure 1D). Cluster 5 did not reflect a presently identifiable immune cell population. Old mice had a higher proportion of cells in cluster 2 (T cells) and a lower proportion in cluster 1 (MMs) (Figure 1C), consistent with our prior observations in young and old mice 15.

The MMs (cluster 1) expressed several homeostatic microglial genes 25 including C1qa, C1qb, C1qc, C3ar1, Cx3cr1, Fcgr1, Mafb, P2ry6 and Trem2 (highlighted red) and genes in common with other peripheral nerve-associated macrophages (NAMs) 18, 19 including Cbr2, Ccl12, and Ms4a7 (highlighted blue) (Figure 1D). Expression of these genes was similarly enriched in MMs from both young and old mice (Supplemental Figure 1A). Similar to other peripheral NAMs 18, 19, MMs did not exhibit increased expression of Sal1 (Supplemental Figure
1B), a transcription factor important for microglial function\textsuperscript{30}, suggesting that while similar to microglia, MMs maintain a genetic signature unique to their microenvironment.

To validate our single cell transcriptomics data, CD45\textsuperscript{+}F4/80\textsuperscript{+}CD11b\textsuperscript{+} cells isolated from SI muscularis (MMs), SI mucosa ([lamina propria macrophages (LpMs)], and spinal cord (microglia) of young mice (3 months, n=9) (Figure 1E) were evaluated for expression of several homeostatic microglial genes. Expression of the microglial purinergic receptor P2ry12\textsuperscript{25} was significantly higher (p<0.001) in MMs compared to LpMs by cell surface and RNA expression (Figure 1F, 1G). MMs also had a significantly reduced (p<0.001) expression of CD45 compared to LpMs (Figure 1F), further reflecting their similarity to microglia, which have low levels of CD45 expression\textsuperscript{31}. Expression of the homeostatic microglial genes Trem2, Cx3cr1, Gpr34, Mef2a, and Hexb was significantly higher (adj p<0.001) in MMs than LpMs in both young and old (26 months, n=6) mice (Figure 1G, Supplemental Figure 1C), consistent with prior reports of microglial-specific gene expression in subsets of gut macrophages\textsuperscript{14, 32, 33}. To determine if this microglial genetic signature was unique to small intestines, homeostatic microglial gene expression was compared in MMs from SI and colon. Except for Trem2, which was elevated in MMs from colon compared to SI, the expression profile of homeostatic microglial genes did not differ (Supplemental Figure 1D), suggesting this microglial genetic signature is common to MMs throughout the gut. To evaluate if sex influences expression of microglial genes, SI MMs were compared between male and female mice (3 months, n≥5/group). MMs from female mice exhibited higher expression of several homeostatic microglial genes compared to males, including P2ry12, Trem2, Cx3cr1, Gpr34, and Mef2a (Supplemental Figure 1E). Collectively,
these data indicate that MMs from SI and colon of both sexes share a genetic signature with microglia and peripheral NAMs.

**MMs acquire a microglial DAM-like phenotype with aging**

To examine the contribution of aging to MM gene expression, scRNA-seq data from MMs of 3- and 24-month old male mice were subjected to unbiased sub-cluster analysis using K-means algorithm. In both age groups, the MM cluster (cluster 1, Figure 1A) segregated into two sub-clusters (1A and 1B) (Figure 2A), but the 1B/1A ratio (calculated per mouse) was significantly higher (p=0.03) in the old than the young mice (Supplemental Figure 2A). Volcano plot analysis (Figure 2B) indicated that sub-cluster 1A was enriched for tissue protective/homeostatic microglial genes relative to sub-cluster 1B, including *Mrc1* (CD206), *Stab1, Maf, Gas6, Fcrls*, and *Gpr34* 21, 25. By contrast, sub-cluster 1B was enriched for several DAM markers, including *Cd9, Itgax* (CD11c), *Lgals3, Bhlhe40, and IL-1β* 21, 22. To confirm that this finding was not exclusive to male mice, SI MMs (CD45-F4/80-CD11b+ cells) sorted from young and old mice of both sexes were examined for the expression of DAM markers including *CD9, Itgax, Bhlhe40, IL-1β*, and *Lgals3*. Expression of several of these DAM markers were markedly higher in old compared to young MMs including *CD9, Itgax* (CD11c), *IL-1β* and *Lgals3* which reached statistical significance in both males and females, and *Bhlhe40* in females (Figure 2C). MMs (CD45-F4/80-CD11b+ cells) were also examined for surface expression of DAM markers CD9, CD11c and CLEC7A. Significantly higher expression of all three markers was detected in old compared to young mice in both sexes by mean fluorescence intensity (MFI) (Figure 2D, 2E). To evaluate the kinetics of DAM gene upregulation as a function of age, MMs from young (3 months), mid-aged (10 months), and old (24 months) male mice were examined...
for expression of CD9, CD11c and CLEC7A. These genes increased in an age-dependent manner with lowest levels seen at 3 months of age (p<0.001) (Supplemental Figure 2B). Expression of the innate immunity receptor Trem2 was also higher (p<0.01) in MMs from old compared to young mice (Figure 2F), consistent with its known upregulation in microglia collected from mice with advanced age or neurodegenerative disease\textsuperscript{21, 22}. Similar to microglia\textsuperscript{21}, the kinetics of Trem2 elevation occurred at a later age than CD9, CD11c and CLEC7A with increased expression observed in 24 month-old mice but not 10 month-old mice (Supplemental Figure 2C).

Collectively, the data indicate that old mice have an MM subpopulation with properties like those of DAM microglia.

**The geriatric MM subpopulation defined by CD11c and Clec7A expression have reduced expression of homeostatic genes and phagocytic activity**

Flow cytometry studies confirmed that old mice have a CD11c\textsuperscript{+}CLEC7A\textsuperscript{+} MM subpopulation [Geriatric State (GS) MMs] that is almost absent in young mice (Figure 3A, 3B). The rise in GS MMs corresponded to a decline in CD11c\textsuperscript{+}CLEC7A\textsuperscript{−} [Homeostatic State (HS)] MMs in old mice. To better understand the potential functional significance of this GS subpopulation, MMs from young (3 months) and old (22 months) mice (n\geq5/group) were sorted based on differential surface expression of CD11c and Clec7A. QPCR analysis comparing the CD11c\textsuperscript{+}CLEC7A\textsuperscript{+} (GS) MMs to the CD11c\textsuperscript{+}CLEC7A\textsuperscript{−} (HS) MMs from both young and old mice indicated that GS MMs express significantly lower levels of homeostatic microglial genes\textsuperscript{25}, including $P2ry12$, $Mef2a$, $Gpr34$, and $Cx3cr1$, and tissue protective genes\textsuperscript{15, 34}, including $Mrc1$, $Clec10a$, and $IL10$, and higher levels of the DAM genes $CD9$, $Itgax$, $Bhlhe40$, $IL1\beta$, and $Lgals3$ than HS MMs (Figure 3C). Sympathetic neurons regulate MM homeostatic function via
signaling through the β2-adrenergic receptor (β2-AR)\textsuperscript{1,6,7}. GS MMs also had lower gene expression of β2-AR (Adrb2) compared to HS MMs (Figure 3C), suggesting that GS MMs have diminished responsiveness to extrinsic signals.

Compared to HS MMs from young and old mice, GS MMs exhibited significantly higher protein and mRNA expression of CD22, a canonical B cell receptor (Figure 3D). Increased CD22 expression in microglia of geriatric mice results in impairment of their homeostatic phagocytic function\textsuperscript{35}. To evaluate the phagocytic activity of MMs, pH-sensitive beads (pHrodo-labeled Zymosan BioParticles) were injected into the colons of young (2 months) and old (22 months) mice (n≥8/group). 1 hour after injections the tissue was harvested, and flow cytometry performed to assess pHrodo signal in MMs. Although the percent of phagocytic (pHrodo\textsuperscript{+}) MMs did not significantly differ between young and old mice (Supplemental Figure 3A, 3B), the fluorescence intensity of phagocytic MMs was significantly reduced for GS compared with HS MMs (Figure 3E), indicating diminished phagocytic activity in GS MMs. Collectively, the data suggest that age-dependent acquisition of the GS phenotype both drives neuroinflammation and results in the loss of MM function important for ENS homeostasis.

**GS phenotype is associated with intracellular α-SYN and clearance of apoptotic neurons**

Having observed that the GS MM phenotype is defined by the expression of DAM markers, we next asked whether its acquisition might involve the clearance of neuronal debris and apoptotic neurons as previously described for microglia DAM marker development\textsuperscript{22,36}. Aggregates of α-SYN, a protein involved in neuronal synaptic vesicle trafficking\textsuperscript{37,38}, accumulate in dystrophic enteric neurons of geriatric rats\textsuperscript{39,40}. MMs surrounding these dystrophic neurons have been observed to contain α-SYN aggregates\textsuperscript{39}. Two series of experiments were done to
explore whether clearance of α-SYN neuronal debris may be involved in the GS phenotype. First, whole mount sections of SI muscularis tissue from young (3 months) and old (24 months) mice (n≥4/group) were stained with antibodies to α-SYN together with antibodies to MHCII (MMs) and Hu (neurons) (Figure 4A). In a second set of experiments, dissociated cells from muscularis tissue were subjected to intracellular staining with AlexaFluor 488-conjugated α-SYN antibody and the percent of α-SYN+ MMs was assessed by flow cytometry (Figure 4B). In both experiments, the percent of α-SYN+ MMs was significantly higher (p<0.005) in old compared to young mice (Figure 4A, 4B). Back-gating onto total MMs indicated that the α-SYN+ MMs were enriched for co-expression of the GS markers CD11c and CLEC7A (Figure 4C). Also, the α-SYN MFI was significantly higher (p<0.001) in GS MMs than HS MMs from young and old mice (Figure 4D). Since aging is associated with elevated numbers of apoptotic neurons in the myenteric plexus of mice15, we examined whether macrophage clearance of apoptotic neurons is a function associated with the geriatric phenotype. Bone marrow-derived macrophages (BMDMs) were co-cultured with UV irradiated fluorescent enteric neurons (apoptotic; aNeurons) and examined for intracellular fluorescence and expression of GS markers (CD11c and Clec7A). Macrophages that had phagocytosed aNeurons were significantly enriched (p<0.05) for CD11c and CLEC7A expression (Supplementary Figure 4). Collectively the data indicate that the GS phenotype (CD11c+Clec7A+) is associated with intracellular accumulation of α-SYN and clearance of apoptotic neurons.

**Human MMs exhibit age-dependent acquisition of the GS phenotype**

Changes in the immune composition of human colon muscularis with age have been poorly explored. Surgical colon specimens were collected from 26 patients whose age ranged
from 25 to 95 years (median=68, Interquartile Range=30.5). 73.1% (19/26) of patients were female and indications for surgery are listed in Table 1. After dissecting the muscle layer and enzymatically digesting the tissue, flow cytometry was performed to assess levels of total leukocytes (CD45+), MMs (CD45+CD14+CD3-CD19+), lymphocytes (CD45+CD14-CD3+CD19+) and dendritic cells (DCs) (CD45+CD14-CD11c+) (Supplemental Figure 5A, 5B). Age was associated with a modest increase in leukocytes (expressed as % of live cells) but the correlation was not significant (Supplemental Figure 5B). Measured as a percent of CD45+ cells, MMs increased (R²=0.05, p=0.28) and lymphocytes declined (R²=0.05, p=0.27) with age while DCs remained stable (Supplemental Figure 5C). Human MMs consist of several distinct subpopulations defined by unique genetic expression profiles. To help define macrophage subpopulations, MMs (CD14+ cells) were also assessed for surface expression of monocyte/macrophage markers CD11b and CD64. Three MM subpopulations were identified, MM₁ (CD11b+CD14highCD64+), MM₂ (CD11b+CD14highCD64-) and MM₃ (CD11b+CD14int) (Figure 5A). Age was associated with an increase in the MM₁ subpopulation (R²= 0.13, p=0.06) and decline in the others (Figure 5B). Expression of known MM markers CD206 (MRC1) and ADRB2₁, ₆, ₁₅ (Figure 5C) and homeostatic microglial genes, including P2RY12, TREM2, CX3CR1, GPR34, and C1QC (Figure 5D), was higher in the MM₁ compared to MM₂ and MM₃ subpopulations, suggesting that MM₁ macrophages represent a NAM subpopulation analogous to murine MMs (Figure 1). Next, we examined whether age was associated with a geriatric MM phenotype like that observed in mice (Figure 2 and 3). Immunostaining of colon tissue sections with antibodies to CD14 and the GS marker CLEC7A (Dectin-1) demonstrated co-labeling of MMs (Figure 5E). Flow cytometry revealed a statistically significant correlation between increasing CLEC7A surface expression and age.
(R²=0.15, p=0.046) in the MM₁ but not MM₂ or MM₃ subpopulations (Figure 5F). Within the MM₁ subpopulation, GS (CD11c⁺CLEC7A⁺) and HS (CD11c⁻CLEC7A⁻) MMs were identified (Figure 5G) and the proportions of GS MMs rose (R²=0.16, p=0.10) and HS MMs declined (R²=0.18, p=0.07) as a function of age (Figure 5G). QPCR analysis of sorted MM subpopulations revealed an age-dependent increase in other GS genes including IL1B (R²=0.08, p=0.2) (Figure 5H), TREM2 (p=0.002) (Figure 5I), ITGAX (R²=0.05, p=0.31) (Supplemental Figure 5D), and CD22 (p=0.01) (Supplemental Figure 5E) exclusive to the MM₁ subpopulation. Collectively, the data indicate that aging results in an expansion of human MMs, particularly the MM₁ subpopulation, which has a genetic signature common with NAMs, and this subpopulation acquires a geriatric phenotype similar to mice.

**α-SYN MM accumulation is associated with the GS phenotype in humans**

An age-dependent increase in α-SYN deposition has been observed in the human ENS⁴¹, however, whether it accumulates in MMs and alters their phenotype is unknown. Colon tissue sections were incubated with antibodies to α-SYN, PGP9.5 (neurons) and CD14 (macrophages). Abundant α-SYN was detected in enteric neurons within the myenteric plexus (Figure 6A) and co-localized to CD14⁺ MMs (arrows) bordering the enteric ganglia (Figure 6B). Intracellular staining with AlexaFluor 488-conjugated α-SYN antibody was performed on dissociated colonic muscularis tissue and α-SYN signal in the MM subpopulations was assessed by flow cytometry (Figure 6C). Higher α-SYN levels were found in MM₁ compared to MM₂ and MM₃ macrophages, consistent with MM₁ macrophages serving a role in clearance of neuronal debris (Figure 6C). Importantly, GS (CD11c⁺CLEC7A⁺) MM₁ macrophages demonstrated significantly higher α-SYN signal compared to HS (CD11c⁻CLEC7A⁻) MM₁ macrophages (Figure 6D). Back-gating onto total
MM₁ macrophages indicated that MMs with high levels of α-SYN (α-SYN²⁻²⁴ MM₁) (Figure 6D) were enriched for co-expression of the GS markers CD11c and CLEC7A (Figure 6E). Collectively, the data suggest that increasing intracellular accumulation of α-SYN causes MM₁ macrophages to acquire a geriatric phenotype.

Discussion

The salient feature of the data presented in this report is the finding of an age-dependent MM genetic signature in a subset of macrophages that mirrors the microglia DAM phenotype. This geriatric phenotype, observed in both mouse and human, is associated with intracellular accumulation of α-SYN and characterized by loss of homeostatic function and increased inflammatory activation. The following comments are pertinent with respect to these findings.

Enteric neurons are vulnerable to age-related degeneration and play an important role in gastrointestinal dysfunction in elderly individuals¹⁵, 42. However, the role of ENS immune cells in the gut dysfunction seen in the elderly is unknown. Our studies were designed to address this question. Using single cell transcriptomics of CD45⁺ cells from the SI muscularis we identified 5 distinct immune cell clusters that respectively represent MMs, T cells, immature and mature B cells, and an unassigned population. We did not identify mast cells or dendritic cells previously seen at low levels in the gut muscularis¹⁵, 43 and do not exclude the possibility that our clusters contain low numbers of other unidentified cell types.

Our studies focused on the MM cell population (cluster 1), because MM functional alterations have been previously implicated in gut dysmotility¹-⁶, 8-¹⁵. Specifically, we asked whether MMs regulate age-dependent gut dysfunction through properties similar to those used
by microglia in neurodegenerative processes. Indeed, microglia express “sensome” genes that include pattern recognition receptors, Fc receptors, chemokine and cytokine receptors, purinergic receptors, and receptors involved in cell adhesion\textsuperscript{44} and are tailored for their specialized support role in the CNS\textsuperscript{20}. While some components of this sensing apparatus are shared among all macrophages, including Toll-like and Fc receptors\textsuperscript{45}, many of them are unique or highly enriched in microglia and peripheral NAMs\textsuperscript{18, 19, 25}. Among these are P2ry12, C1q, Gpr34, Csf1r and Cx3cr1, which are involved in microglia housekeeping and neuroprotection\textsuperscript{25, 46-49}. For example, microglia directly sense neuronal mitochondrial activity through the purinergic receptor P2ry12, enabling a rapid neuroprotective response to neuronal injury\textsuperscript{46}. Gpr34, another member of the P2ry12 receptor family, and C1q, a component of the complement system, regulate microglial phagocytic function and removal of neuronal debris\textsuperscript{47, 48, 50}. Also, through the Cx3cr1 receptor, microglia sense the neuronally expressed chemokine fractalkine to regulate synaptic pruning\textsuperscript{49}.

We found that MMs, similar to other peripheral NAMs\textsuperscript{18, 19}, express several microglial homeostatic genes including P2ry12, C1q and Gpr34, as well as Csf1r and Cx3cr1\textsuperscript{2, 14}, suggesting they function to sense environmental perturbations and provide housekeeping support to the ENS, as is the case for microglia. Supporting the important role these genes have in ENS homeostasis, macrophage specific deletion of C1qa results in changes in enteric neuron gene expression and altered intestinal motility\textsuperscript{51}. We found the microglial genetic signature in MMs of both mouse and human, males and females, and SI and colon. Interestingly, many of the microglial homeostatic genes were expressed higher in female compared to male mice.
(Supplemental Figure 1E). Whether similar sex differences exist in humans and if these differences have functional consequences are the subject of ongoing investigation.

Comparison of old to young mice indicated that MMs express similar levels of homeostatic microglial genes, but expression of GS genes, which mirror the microglial DAM phenotype, is acquired with aging. In microglia, the DAM phenotype is believed to represent the compensatory upregulation of a neurodegeneration-associated molecular pattern (NAMP) apparatus, which initially has a protective role that increases neuronal debris clearing but eventually drives neuroinflammation\textsuperscript{52}. While it remains unclear whether the GS phenotype has a protective role at an early stage, we found that the MM GS phenotype is associated with increased expression of the pro-inflammatory cytokine IL-1β (Figure 2B and 3C) and reduced expression of the anti-inflammatory cytokine IL-10 (Figure 3C). GS MMs also exhibited increased CD22 expression that, similar to microglia\textsuperscript{35}, resulted in loss of phagocytic activity (Figure 3E). GS MMs also had reduced expression of Adrb2 (Figure 3C), the adrenergic receptor important for MM mediated ENS recovery following enteric infections\textsuperscript{1, 6, 7}. This offers a potential explanation for why elderly individuals have diminished resilience to enteric infections like *Clostridium difficile*\textsuperscript{53}. Collectively, our data suggest that the GS phenotype has a deleterious effect on ENS homeostasis by promoting neuroinflammation and accumulation of degenerative debris\textsuperscript{15}.

Consistent with previous findings that phagocytosis of neuronal debris promotes the microglial DAM phenotype\textsuperscript{22, 36}, we found that GS, but not HS macrophages, contain high levels of α-SYN aggregates suggesting that phagocytosis of neuronal debris also promotes acquisition of the GS phenotype. Our findings may have relevance to the pathogenesis of
neurodegenerative disorders in the gut. α-SYN aggregation, the hallmark pathogenic factor in Parkinson’s disease (PD), is found in the gut of PD patients and emerging evidence suggests the disease initiates in the gut and is transferred centrally through the vagal pathway\textsuperscript{54}. Elevation in enteric α-SYN is associated with increased gut inflammation and delayed intestinal transit in PD patients and animal models\textsuperscript{55, 56}. We propose that PD and other neurodegenerative disorders accelerate the accumulation of GS macrophages, thereby causing ENS neuroinflammation and disruption of gut function. Additionally, loss of HS MMs could also contribute to disease pathogenesis. Homeostatic microglia play a role in clearing α-SYN aggregates via autophagy\textsuperscript{57}. Therefore, declining numbers of HS MMs in old mice could explain why in a PD animal model, after α-SYN gut injections, brain transmission was observed in old but not young mice\textsuperscript{55}. Further clarification of the role of gut macrophages in PD and other neurodegenerative disorders is necessary to determine whether targeting the GS immunophenotype may be effective in treating these disorders.

We conclude that human MMs acquire a GS phenotype similar to that of mice based on higher proportions of CD11c\textsuperscript{+}CLEC7A\textsuperscript{+} MM\textsubscript{1} macrophages (Figure 5G) and increased expression of Trem2 and CD22 with age (Figure 5I, Supplemental Figure 4E). However, our findings in humans and mice were not entirely equivalent. Correlations between age and GS gene expression did not reach statistical significance for IL1B (R\textsuperscript{2}=0.08, p=0.2) (Figure 5H) or ITGAX (R\textsuperscript{2}=0.05, p=0.31) (Supplemental Figure 5D). A potential explanation is the heterogeneity of our human samples which differed based on surgical indications. Notably, exclusion of the two specimens with the highest leukocyte infiltration [corresponding to patients with adhesive mesh and perforated diverticulitis (Supplemental Figure 4B)] resulted in better correlation
between *IL1B* expression and age (p=0.07). This suggests that certain inflammatory conditions may mask the effects of age. Additional limitations to our study include low numbers of young patients (only one patient <40 years of age) and male subjects (7/26). Future studies involving greater numbers of patients and functional measures of gut motility are necessary to determine whether the GS phenotype may serve as a biomarker or therapeutic target for age-related GI disorders.

In conclusion, our studies document, for the first time, that MMs function as the ENS microglia, serving an important housekeeping and neuroprotective role. They share a common genetic signature with microglia and peripheral NAMs, which enables environmental sensing and communication with enteric neurons, and acquire a geriatric state with aging that reduces their functional efficacy resulting in neuroinflammation and disruption of gut motility.

**FIGURE LEGENDS**

**Figure 1. Transcriptional profile of CD45\(^+\) cells in small intestine muscularis.**

A. Schematic diagram of CD45\(^+\) cell isolation from small intestine (SI) muscularis (modified from Furness and Costa, 1980).  
B. t-SNE plot of the scRNA-seq analysis of 1,705 isolated CD45\(^+\) cells using the drop-seq method identifies 5 cell clusters in both young (3 months, n=2) and old (24 months, n=3) C57BL/6 mice.  
C. Stacked bar graph shows the percent of CD45\(^+\) cells found in Clusters 1-5 in young and old mice from the data defined in panel B.  
D. Heatmap of differentially expressed genes that define clusters in panel B. Genetic phenotypes for cell classification are in bold font and asterisked. Genes in common with microglia\(^{25}\) and peripheral nerve-associated macrophages\(^{18,19}\) are highlighted in red and blue, respectively.  
E.
Representative flow cytometric dot plots of F4/80⁺CD11b⁺ cells isolated from SI muscularis (muscularis macrophages (MMs)), SI mucosal layer [lamina propria macrophages (LpMs)], and spinal cord (microglia). Numbers on plot are percent of CD45⁺ cells (mean ± SD). F. P2ry12 and CD45 expression in LpMs, MMs, and microglia was measured by flow cytometry and is expressed as mean fluorescence intensity (MFI). G. Expression of homeostatic microglial genes P2ry12, Trem2, Cx3cr1, Gpr34, Mef2a, and Hexb in sorted LpMs and MMs was measured by qPCR analysis and results are expressed as relative expression, calculated using the Pfaffl method. Data are mean ± SEM. *p<0.05, ***p<0.005, ****p<0.001 as determined by one-way analysis of variance with Bonferroni’s multiple comparisons (F) and multiple comparisons t-test with post-hoc two-stage linear step-up procedure (G).

**Figure 2. MM expression of DAM-like markers increases with age.** A. t-SNE plot of scRNA-seq data for cluster 1 cells (MMs) identifies two sub-clusters (1A and 1B) with sub-cluster 1A (red) decreasing and sub-cluster 1B (blue) increasing in old (24 months) compared to young (3 months) mice. B. Volcano plot shows log₂ fold change of genes in sub-cluster 1A/1B by significance (-log₁₀ FDR adjusted p). DAM²¹,²² and homeostatic microglial genes²⁵ are highlighted in blue and red, respectively. C. Expression of DAM genes CD9, Itgax (CD11c), Bhlhe40, Il1β, and Lgals3 by qPCR analysis in sorted MMs (F4/80⁺CD11b⁺ cells) from SI of young (3 months), and old (16-29 months) male and female mice (n≥5 each). Results are presented as relative expression normalized to young male SI MMs. D. Representative flow cytometric surface staining of DAM markers CD9, CD11c (Itgax) and CLEC7A on MMs from young (red) and old (blue) mice. Gray histograms represent fluorescence minus one (FMO) control. E. Results from (D) are expressed as MFI for male and female mice. F. Trem2 mRNA levels in MMs of
male mice measured by qPCR and results are expressed as relative expression. Data are mean ± SEM. *p<0.05, ***p<0.005, ****p<0.001 by multiple comparisons t-test with post-hoc two-stage linear step-up procedure.

**Figure 3. GS MMs have reduced expression of homeostatic microglia and tissue-protective genes.**

**A.** Representative flow cytometric dot plots of F4/80^+^CD11b^+^ MMs from young and old male mice (n=5 each) analyzed for CD11c and CLEC7A surface expression demonstrate a CD11c^-^CLEC7A^-^ homeostatic state (HS) subpopulation present in both young (red box) and old mice (pink box) and a CD11c^-^CLEC7A^+^ subpopulation (blue boxes) present predominantly in old mice [geriatric state (GS)]. Numbers on plot represent percent of MMs (mean ± SD).

**B.** The proportion of HS (CD11c^-^CLEC7A^-^) and GS (CD11c^-^CLEC7A^+^) MMs in young and old mice expressed as percent of MMs.

**C.** Sorted GS MMs from old (blue) mice and HS MMs from young (red) and old (pink) mice (n≥5 each, male) were examined for expression of homeostatic state (HS) genes *P2ry12, Mef2a, Gpr34, Cx3cr1, Mrc1, Clec10a, IL10,* and *Adrb2,* and geriatric state (GS) genes *CD9, Itgax, Bhlhe40, IL1β,* and *Lgals3* by qPCR and results are shown as relative expression.

**D.** GS and HS MM were also examined for CD22 expression by flow cytometry with Alexa Fluor-488-labeled CD22 antibody (protein) and qPCR (mRNA). Results are respectively expressed as mean fluorescence intensity (MFI) and relative expression.

**E.** HS (CD11c^-^CLEC7A^-^) and GS (CD11c^-^CLEC7A^+^) colon MMs were assessed for green fluorescence 1 hour after pHrodo Green Zymosan Bioparticles were injected into the colon muscularis of young (2 months) and old (22 months) mice (n≥8 each, male). Results are expressed as MFI of the phagocytic (pHrodo^-^) MMs. Data are mean ± SEM. *p<0.05, **p<0.01, ***p<0.005, ****p<0.001 by two-way analysis of variance with Bonferroni’s multiple comparisons (B) or with post-hoc two-stage
linear step-up procedure (C), and one-way analysis of variance with Bonferroni’s multiple comparisons (D, E).

**Figure 4. MMs acquire the GS phenotype through clearance of α-SYN neuronal debris.** A. SI muscularis tissue from young (3 months) and old (23-28 months) mice (n≥4 each) were stained with antibodies to α-SYN (red), Hu (pan-neuronal marker, blue) and MHCII (macrophage marker, green) and a minimum of 8 randomly selected fields containing myenteric ganglia were examined by confocal microscopy. α-SYN aggregates (arrowheads) and MMs containing α-SYN (arrows) are shown. The % α-SYN+ MMs were significantly higher in old than young mice. B. Representative flow cytometric histograms of intracellular immunofluorescent staining of MMs with Alexa Fluor-488-labeled α-SYN antibody in old and young mice (n=6 each). FMO (grey histogram) was used to determine the cutoff (black box) for defining α-SYN+ MMs and results are expressed as % α-SYN+ MMs. C. When backgated onto MMs (grey), α-SYN+ MMs (red) were enriched for cells co-expressing GS markers CD11c and CLEC7A (representative image of two independent experiments). D. The α-SYN levels expressed as MFI in GS (CD11c+CLEC7A+) and HS (CD11c CLEC7A-) MMs. Data are mean ± SEM. ***p<0.005 by t-test (A and B) and one-way analysis of variance with Bonferroni’s multiple comparisons test (D). Scale bars 25 μm.

**Figure 5. Human MMs have a NAM genetic signature and acquire a geriatric phenotype with age.** A. Representative flow cytometric dot plots demonstrating gating scheme for human MMs. CD45+ cells isolated from the muscularis layer of human colon were examined for surface expression of monocyte/macrophage markers CD14, CD11b and CD64. Three subpopulations of MMs were identified based on intensity of marker expression: MM1 (CD11b+CD14highCD64+, green), MM2 (CD11b+CD14highCD64-, yellow) and MM3 (CD11b+CD14int,
orange). Numbers on plot are percent of CD14+ cells (mean ± SD). B. Relationship between proportions of MM subpopulations [expressed as % total MMs (CD14+)] and age in years. Regression lines and Pearson correlation statistics (for MM1) are shown. C. MM subpopulations were examined for CD206 (MRC1) and ADRB2 expression by flow cytometry and qPCR. Results are expressed as mean fluorescence intensity (MFI) and relative expression. D. Expression of microglial homeostatic genes P2RY12, TREM2, CX3CR1, GPR34, and C1QC by qPCR analysis in MM subpopulations. Results are presented as relative expression normalized to MM1 macrophages. E. Colon tissue section (80yo F) stained with antibodies to CLEC7A (red) and CD14 (macrophage marker, green) and DAPI (blue) was examined by a Keyence Fluorescence Microscope. MMs expressing CLEC7A are shown (arrows). F. Relationship between surface expression of CLEC7A in MM subpopulations as measured by flow cytometry (expressed as MFI) and age (in years). Regression lines and Pearson correlation statistics (for MM1) are shown. G. Representative flow cytometric dot plots of MM1 macrophages from a young (25yo F) and old (92yo F) patient analyzed for CD11c and CLEC7A surface expression with gates defining HS (CD11c−CLEC7A−) and GS (CD11c+CLEC7A+) MM1. Relationship between proportions of GS/HS MM1 subpopulations (expressed as % total MM1) and age in years. Regression lines and Pearson correlation statistics are shown. H. Relationship between IL1B expression in sorted MM subpopulations as measured by qPCR and age (in years). Regression lines and Pearson correlation statistics (for MM1) are shown. I. Expression of Trem2 in MM subpopulations by qPCR analysis from patients 25-74 years of age and ≥75 years of age. Data are mean ± SEM. *p<0.05, **p<0.01, ***p<0.005, ****p<0.001 by one-way analysis of variance.
with Bonferroni’s multiple comparisons test (C), two-way analysis of variance with post-hoc two-stage linear step-up (D) or Bonferroni’s multiple comparisons test (I). Scale bar 50 μm.

**Figure 6. α-SYN accumulation in human MMs associated with geriatric phenotype.**

A. Colon tissue section (82yo F) stained with antibodies to PGP9.5 (neuron marker, green) and α-SYN (red) and DAPI (blue) examined by a Keyence Fluorescence Microscope.  
B. Colon tissue section (84yo M) stained with antibodies to CD14 (macrophage marker, green) and α-SYN (red) and DAPI (blue). Co-localization of α-SYN with MMs are shown (arrows).  
C. Representative flow cytometric histograms of intracellular immunofluorescent staining of α-SYN in MM subpopulations (grey histogram is FMO). The α-SYN levels in MM$_1$, MM$_2$, MM$_3$ macrophages are expressed as MFI.  
D. Representative flow cytometric histograms of α-SYN signal in HS (CD11c$^-$CLEC7A$^-$, white) and GS (CD11c$^+$CLEC7A$^+$, red) MM$_1$ macrophages (grey histogram is FMO). Dotted black line is the cutoff for defining α-SYN$^{Hi}$ MMs. The α-SYN levels in HS and GS MM$_1$ macrophages are expressed as MFI.  
E. When backgated onto total MM$_1$ (grey), α-SYN$^{Hi}$ MM$_1$ macrophages (red) were enriched for cells co-expressing GS markers CD11c and CLEC7A. Data are mean ± SEM. ****p<0.001 by t-test (D). Scale bars 50 μm.

**REFERENCES**


Table 1. Patient Characteristics

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CA, cancer; STC, slow transit constipation; XRT, radiation therapy
Patient 6 had surgery twice for rectal prolapse
A

Myenteric plexus

Submucosal plexus

B

Cell Clusters

Cluster 1 (MMs)
Cluster 2 (T cells)
Cluster 3 (Immature B cells)
Cluster 4 (Mature B cells)
Cluster 5 (Unidentified)

t-SNE1

t-SNE2

C

Percent of CD45

Young
Old

D

Cluster 1
Cluster 2
Cluster 3
Cluster 4
Cluster 5

Homeostatic Microglial Genes

E

P2ry12

MFI

Fourelix

MMs

Microglia

G

Relative Expression

LpMs
MMs

Homeostatic Microglial Genes

Journal Pre-proof