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Gene expression changes in aging retinal microglia: relationship to microglial support functions and regulation of activation

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Abstract

Microglia, the resident immune cell of the central nervous system (CNS), are thought to contribute to the pathogenesis of age-related neurodegenerative disorders. It has been hypothesized that microglia undergo age-related changes in gene expression patterns that give rise to pathogenic phenotypes. We compared the gene expression profiles in microglia isolated *ex vivo* from the mouse retinas of ages ranging from early adulthood to late senescence. We discovered that microglial gene expression demonstrated progressive change with increasing age and involved genes that regulate microglial supportive functions and immune activation. Molecular pathways involving immune function and regulation, angiogenesis, and neurotrophin signaling demonstrated age-related change. In particular, expression levels of complement genes, *C3* and *CFB*, previously associated with age-related macular degeneration (AMD), increased with aging, suggesting that senescent microglia may contribute to complement dysregulation during disease pathogenesis. Taken together, senescent microglia demonstrate age-related gene expression changes capable of altering their constitutive support functions and regulation of their activation status in ways relating to neuroinflammation and neurodegeneration in the CNS.

Keywords

aging; microglia; retina; microarray; gene expression; complement; activation; angiogenesis; neurotrophic factors; senescence

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1. Introduction

Advancing age is strongly associated with the increasing prevalence of neurodegenerative disease of the retina, such as age-related macular degeneration, glaucoma, and diabetic retinopathy (Friedman *et al.* 2004a; Friedman *et al.* 2004b), which constitute the leading causes of low vision and legal blindness in the developed world (Congdon *et al.* 2003; Congdon *et al.* 2004). While the pathogenic mechanisms for these age-related retinal diseases remain unclear, chronic neuroinflammation resulting from the activation of the immune system features prominently (Wax & Tezel 2009; Buschini *et al.* 2011; Tang & Kern 2011) and appears to be causally related to disease progression. Histopathological specimens from affected humans (Yuan & Neufeld 2001; Gupta *et al.* 2003; Zeng *et al.* 2008) and from animal models of disease (Krady *et al.* 2005; Combadiere *et al.* 2007; Bosco *et al.* 2011) demonstrate the early involvement of retinal microglia, implicating them as an initiating source of neuroinflammatory change underlying disease pathogenesis.

The common elements in aging and microglial changes in neurodegenerative disease suggest that senescent changes in microglia may play a causal role in pathogenic neuroinflammation (Streit & Xue 2009; von Bernhardi *et al.* 2010). Recent studies utilizing the technique of parabiosis to create chimerism in bone-marrow derived precursors have revealed that microglia indeed have long tenures in the course of an animal's regular life span in the undiseased CNS (Ajami *et al.* 2007; Mildner *et al.* 2007). The resulting low turnover rate of microglia *in situ* indicates their susceptibility to senescence-related changes, which can influence the aging CNS milieu in potentially pathogenic ways.

There is accumulating evidence that microglia can exhibit phenotypic changes with advancing organismal age. Microglia have a unique phenotype in the uninjured CNS by virtue of their highly ramified morphology and rapidly and continuously moving processes, which allow their constant contact with neighboring neurons and glia (Davalos *et al.* 2005; Nimmerjahn *et al.* 2005; Lee *et al.* 2008). These dynamic and repeated cell-cell contacts are thought to subserve constitutive functions of synapse regulation and neuronal support (Paolicelli *et al.* 2011; Schafer *et al.* 2012b; Vinet *et al.* 2012). We and others have previously shown that phenotypic features of microglia undergo senescent change in which aged microglia become less ramified and move their processes with decreased dynamism (Sierra *et al.* 2007; Damani *et al.* 2011; Tremblay *et al.* 2012), suggesting a decline in their supportive functions with aging. In addition, aged microglia demonstrate dysregulation in their activation status. Microglia in aged brains show increased signs of activation at baseline (Perry *et al.* 1993; Sheng *et al.* 1998) and respond to activating triggers in a manner that is more augmented and prolonged compared to microglia in young brains (Xie *et al.* 2003; Sierra *et al.* 2007). In the retina, we have shown that aging microglia, in accumulating increased intracellular lipofuscin, exhibit dysregulated complement activation and increased secretion of inflammatory cytokines (Ma *et al.* 2013). These findings indicate that microglia are susceptible to a senescent loss of proper regulation in activation in affected tissues.

Molecular mechanisms underlying age-related phenotypic changes in microglia are yet unclear. We investigate this question in the current study by comparing gene expression patterns in microglia isolated *ex vivo* from mouse retinal tissue obtained from age groups spanning the full range of adult aging. We have focused on microglia located in the retina, a specialized division of the CNS, though findings here may potentially be generalized to microglia elsewhere (de Haas *et al.* 2008). Analyses of age-related gene expression in the whole retina has been previously performed (Yoshida *et al.* 2002; Chen *et al.* 2008a), identifying genes involved inflammatory responses (Chen *et al.* 2010; Van Kirk *et al.* 2011) and implicating immunological influences in the overall aging phenotype of the retina (Xu *et al.* 2009). However, individual contributions of different retinal cell types cannot be

discerned in these studies. The current study represents an advance on previous work in its specific analysis of retinal microglia isolated *ex vivo*, which allows the specific contribution of microglial aging changes to be separately examined.

Our results demonstrate that the overall profile of gene expression in retinal microglia changes as a function of aging and highlight particular molecular pathways regulating microglial constitutive function, microglial activation, and complement regulation. These changes may relate to the development of known senescent microglial phenotypes, including those related with pathological significance. These associations can help direct further investigation into molecular mechanisms underlying age-related immune dysregulation and the search for anti-inflammatory therapeutic approaches to age-related retinal disease.

2. Methods

2.1 Experimental animals of different age groups

Wild type male C57BL/6J mice of four age groups (3 months, 12 months, 18 months, and 24 months) spanning the entire period from adulthood to late senescence were obtained from the NIA Aged Rodent Colonies (National Institutes on Aging, Bethesda, MD, USA). Heterozygous CX3CR1$^{+/gfp}$ transgenic animals, created by breeding CX3CR1$^{gfp/gfp}$ mice (Jung *et al.* 2000)(The Jackson Laboratory, Bar Harbor, ME) with wild type mice, were used for immunohistochemical studies. All animals were genotyped for the rd8 mutation in the Crb1 gene, a mutation recently found in lines of inbred and transgenic mice (Mattapallil *et al.* 2012), and were confirmed to lack this mutation. Briefly, detection of the rd8 mutation versus the wild type genotype was performed using two genotyping methods: 1) by PCR using a TaqMan allelic discrimination assay, and 2) by DNA sequencing of the rd8-associated nucleotide deletion using methods and PCR primers as previously described (Mattapallil *et al.* 2012). All animals were housed in a National Institutes of Health animal facility under a normal 12-hour light-dark cycle. Experiments were conducted according to protocols approved by a local Institutional Animal Care and Use Committee and adhered to the Association for Research in Vision and Ophthalmology (ARVO) Statement for the use of animals in ophthalmic and vision research. Both retinas of each mouse were combined as a single biological replicate; at least four animals from each age-group were used.

2.2 Isolation of retinal microglia

Mice were euthanized and the eyes were immediately enucleated. The globes were immersed in ice-cold Hank's balanced salt solution (HBSS), the retinas were isolated by dissection and transferred into 0.2% papain solution including glucose (1mg/ml), DNAse1 (Worthington, Lakewood, NJ, 100U/ml), superoxide dismutase (SOD) (Worthington, 5μg/ml) and catalase (Sigma, St Louis, MO, 5μg/ml) in HBSS, and incubated at 8°C for 45 min and then at 28°C for 7 min. The digested tissue was dissociated by trituration and centrifuged at 150G for 5 min at 4°C. The resulting cell pellet was resuspended with neutralization buffer containing glucose (2mg/ml), DNAse1 (100U/ml), SOD (5μg/ml), catalase (5μg/ml), antipain (Roche, Indianopolis, IN, 50μg/ml), d-alpha-tocopheryl acetate (Sigma, 10μg/ml), albumin (40mg/ml), and gentamycin (Sigma, 1μl/ml), and again centrifuged at 150G for 5 min at 4°C. The cellular pellet was resuspended in 100μl of staining buffer (BD Pharmingen, San Diego, CA, Cat#: 554656) containing a FITC-conjugated antibody to CD11b (1:50, eBioscience, San Diego, CA, Cat#11–0112), and incubated for 30 minutes at 4°C to label retinal microglia. The cells were washed twice in 10ml of staining buffer containing 2mM EDTA and suspended with 0.5ml of staining buffer. Labeled retinal microglia were isolated by fluorescence-activated cell sorting (FACS) (BD FACSAria II Flow Cytometer, BD, Franklin Lakes, NJ) at the NEI Flow Cytometry Core Facility (Supplementary Fig 1A). Viability of sorted cells was assessed by labeling with

Cy-5-conjugated propidium-iodide; mean percentage of live cells to whole cells was 86.0±4.0%, with no significant differences found between samples from animals of different ages. Approximately 1500–1800 CD11b+ microglial cells were obtained from both retinas of each experimental animal. These were collected into a 1.5ml eppendorf tube containing 200μl of RNAprotect reagent (Qiagen, Valencia, CA, Cat#D-40724) and stored at −80°C for subsequent RNA extraction.

The selectivity of the above CD11b-based method for isolating microglia was verified by additional experiments using $CX3CR1^{+/gfp}$ transgenic mice. Immunohistochemical staining in retinal flat-mounts revealed that all GFP+ retinal microglia in $CX3CR1^{+/gfp}$ animals were also immunopositive for CD11b (Supplementary Fig 1B). Flow cytometric cell sorting of retinal microglia from $CX3CR1^{+/gfp}$ animals using the same CD11b-based method demonstrated that cells isolated on the basis of CD11b were simultaneously positive for GFP (Supplementary Fig 1C), indicating that isolated CD11b+ cells were indeed CX3CR1-expressing microglia.

2.3 Total RNA extraction, microarray hybridization, processing and analysis

Total RNA extraction was performed on FACS-sorted microglia cells with RNeasy Mini kit (Qiagen, Valencia, CA) following the manufacturer's instruction. The extracted RNA was quantitatively analyzed (Agilent 2100 Bioanalyzer; Agilent Technologies, Palo Alto, CA); the mean RIN from 16 samples was ≥1. Total RNA (10–500 pg) from purified microglia from each biological replicate was used for target generation. cDNA amplification from the extracted RNA was performed using the Ovation One-Direct RNA amplification (NuGen Technologies, San Carlos, CA) according to the manufacturer's protocol. The amplified cDNA was fragmented and labeled with biotin using the Encore Biotin Module (NuGen Technologies). The labeled target (5 μg) was applied to a GeneChip Mouse Exon 1.0ST Array, hybridized for 21 hours at 45°C, washed and stained on an Affymetrix GeneChip Fluidics Station 450, and then scanned with a GeneChip Scanner 3000 7G (all from Affymetrix, Santa Clara, CA).

Raw data were normalized and analyzed using the GeneSpring GX 11.0.2 software (Silicon Genetics, Redwood City, CA). The Robust Multichip Average (RMA) method (Irizarry *et al.* 2003) was used for background correction, normalization, and summarization of expression scores for gene expression data. A one-way ANOVA was used to compare gene expression levels between consecutive time-points (3 months to 12 months, 12 months to 18 months, 18 months to 24 months). Gene expression changes that were increased or decreased by a factor of greater than 1.5-fold and had an uncorrected p-value of <0.05 in at least one of the comparisons were identified. From this overall set, subsets of genes that demonstrated a "monotonic-to-age" change in expression across the entire ranges of ages were identified for further analysis. Two subsets of genes were identified: (1) genes that increased monotonically with time (i.e. genes that either increased significantly or remained stable between time-points) and (2) genes that decreased monotonically with time (i.e. genes that either decreased significantly or remained stable between time-points). Ingenuity Pathway Analysis (IPA, Ingenuity Systems, Redwood City, CA) was employed to identify affected networks and functional pathways within the gene lists. Microarray data have been deposited into NCBI's Gene Expression Omnibus (GEO; accession number GSE38739) in compliance with Minimum Information About a Microarray Experiment (MIAME) guidelines.

2.4 Real-time quantitative reverse transcriptase polymerase chain reaction (rt-PCR) analysis

Quantitative rt-PCR analysis of FACS-purified retinal microglia cells was performed to validate findings from the microarray data. Retinal microglia from four additional independent biological replicates for each of the four aging time-points (3, 12, 18 and 24 months of age) were obtained as described above. RNA was extracted from microglia (RNeasy Mini kit, Qiagen, Valencia, CA), amplified to cDNA (WT-Ovation™ One-Direct System, NuGEN, San Carlos, CA), and purified (MinElute Reaction Cleanup kit, Qiagen) following the manufacturers' instructions. 2 μl of cDNA was used for real-time PCR using SYBR Green PCR Mastermix on the 7900HT Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA). β-2 microglobulin(B2M) and ribosomal protein S13 served as internal controls. Primer pairs tested are listed in Supplementary Table 1.

2.5 Immunohistochemistry and image analysis

Retinal and sclerochoroidal flatmounts were prepared from CX3CR1$^{+/gfp}$ mice as described previously (Damani *et al.* 2011). Sclerochoroidal flatmounts containing a surface monolayer of retinal pigment epithelial (RPE) cells with adhering subretinal microglia were immunostained with the antibodies to the following antigens: C3 (Hycult Biotech, Clone 11H9, Cat#HM1045, The Netherlands, 1:200), complement factor B (CFB) (Santa Cruz Biotechnology, Cat# sc-67141, 1:200), and C3b/iC3b/C3c (mAb 2/11,#HM1078, Hycult, Uden, The Netherlands, 1:200). Secondary antibodies, conjugated to Alexa-633 (Invitrogen, Carlsbad, CA), were added at a 1:200 dilution and incubated for 1–2 hours. Tissue preparations were mounted onto slides and imaged using confocal imaging with a 60x oil objective at a resolution of 1024×1024 pixels; similar imaging settings were employed in the imaging of tissue of different ages. The extents of immunostaining for antibodies to C3, CFB, and iC3b were quantitated using ImageJ software (National Institutes of Health, Bethesda, MD); maximum intensity projections of z-series confocal images stacks were converted to binarized images using a common thresholding function and counts of immunopositive pixels performed. Quantitative analysis of microglial morphological parameters were performed as previously described (Fontainhas *et al.* 2011). Analysis of microglial soma size was performed by delineating the outline of individual microglia somata using the smooth polygon tool in NIH ImageJ and quantifying the area and perimeter of the circumscribed region. Analysis of microglial dendritic size was performed by circumscribing the area delimited by the termini of dendritic processes of individual microglia and quantifying the area and perimeter of the circumscribed region. Maximum intensity projection image of individual GFP+ microglia was binarized and a topological skeleton derived from the binary image using the "skeletonize" function in ImageJ and total dendritic length was calculated from the skeletonized image.

3. Results

3.1 Profiles of age-associated gene expression in mouse retinal microglial cells

In order to evaluate the differential expression of genes in microglia in the retina, we purified CD11b-positive microglia from the retinas of wild type C57BL/6 mice aged 3, 12, 18, and 24 months (four animals from each age group) using cell sorting with flow cytometry. The number of retinal microglia isolated per experimental animal (using two retinas from each animal) averaged 1805±805 cells (mean±SD). The number of retinal microglia isolated per animal were statistically similar among different age groups ($p > 0.05$ for all comparisons, 1-way ANOVA with Tukey's multiple comparison test). Age-related differential expression of genes from isolated retinal microglia was analyzed using 16 Affymetrix GeneChip Mouse Exon 1.0ST Arrays. Analysis revealed high expression levels of microglia-associated genes (CD11b, Iba1, P2Y12, and CX3CR1) while genes expressed

by macroglia (GFAP), vascular cells (NG2), RPE cells (RPE65), and retinal neurons (calbindin, Brn3a, Brn3b) were expressed at significantly lower background levels (*data not shown*). Differential expression levels of individual genes between consecutive age groups (i.e. between 3 and 12 months, 12 and 18 months, and 18 and 24 months) were examined to identify microglial genes demonstrating significant changes in mRNA expression across the full range of ages ranging from mature adulthood to late senescence.

We identified a total of 719 genes (out of a total of 16711) that showed differential expression of >1.5-fold change at $p<0.05$ (uncorrected one-way ANOVA) for at least one of the 3 age-group comparisons (Supplementary Table 2). This method of selection had the goal of identifying genes that are altered in expression level not only at the "end-stage" of late senescence but throughout the entire period of adult aging, from young adulthood through "middle-age" to late senescence. These differentially expressed genes were subjected to a hierarchical cluster analysis to visualize trends in differential expression across individual biological repeats in the 4 age groups (Fig. 1). Individual biological repeats showed a tendency to cluster within their age-group, demonstrating progressive changes in retinal microglial gene expression with age.

Among the 719 genes showing age-dependent expression changes, two subsets of genes were identified that demonstrated either an increasing or a decreasing "monotonic-to-age" pattern of change (i.e. a gene that showed an increasing "monotonic-to-age" pattern would demonstrate a significant increase in expression across one or more consecutive age-groups without significantly decreasing in expression across any consecutive age-group). Of 719 genes, 295 (41%) genes demonstrated a monotonic increase in expression with increasing age, while 284 (39%) genes demonstrated a monotonic decrease in expression with age. The lists of up-regulated and down-regulated genes from each category respectively are shown in Supplementary Tables 3 and 4.

Real-time quantitative rt-PCR (qRT-PCR) was performed for 20 selected genes on cDNA isolated from similarly FACS-sorted retinal microglia. The analysis demonstrated significant age-related changes in gene expression between the 3-month old and 24-month old age groups that were consistent in direction to that observed from the microarray analysis in 19 out of 20 genes (Fig. 2). Also, "housekeeping" genes (RPS13, RPS 9) and functional microglial genes (CD68, Iba1), which were relatively unchanged in expression on microarray analyses, demonstrated a similar relative stability in expression levels on qRT-PCR analyses (Supplementary Fig. 2).

3.2 Biological features of gene expression pattern changes associated with aging in retinal microglia

Gene ontology (GO) enrichment analysis (Sheehan *et al.* 2008) was used to assign significant age-related gene expression changes to biologically meaningful categories. The GO categories with "Cellular Process" with enrichment scores > 3 and with a representation of at least two differentially-expressed genes were identified and organized according to the broader GO categories in which the subcategories belong (Table 1). In this analysis, genes that demonstrated age-related expression changes involved: 1) biological regulatory mechanisms governing transcription, signal transduction, activity of signaling pathways, phosphorylation, and cell proliferation and growth, 2) lipid biosynthetic process, 3) transport of organic substances and intracellular proteins, and 4) immune system processes, specifically the control of microglial activation.

Ingenuity® Pathway Analysis (IPA) which employs a curated genetic database of functional assignments was used to analyze the full set of 719 differentially-expressed genes to discover canonical pathways in microglia influenced by aging. A list of pathways in which

age-related microglial genes demonstrate significant representation is shown in Table 2. These pathways included those that have been related to: (1) microglial immune function and regulation (IL-17A, IL1, and IL3 signaling, ceramide signaling, nitric oxide signaling, estrogen receptor signaling, and LPS-stimulated MAPK signaling) (Fig. 3), (2) angiogenesis (TSP1 regulated angiogenesis, and VEGF ligand-receptor interactions) (Fig. 4A, B), and (3) trophic factors (neurotrophin/TRK signaling) (Fig. 4C). These associations indicated that the aging microglial phenotype may involve its ability to regulate immune and inflammatory reactions, influence angiogenesis in its environment, and support neuronal survival and functioning.

In order to explore the direct interactions between the 719 differentially expressed genes, we employed MetaCore™ from GeneGo, Inc. (Carlsbad, CA) to generate a network construction of interactions (Supplementary Fig. 3). AP1 and Egr1 were identified as “hubs” with the greatest number of interactions between differentially expressed genes.

3.3 Age-related expression changes in genes regulating complement activation

Among the subset of genes which showed monotonic increasing expression with age (295 genes), analysis of direct interactions highlighted those occurring between *C3* and *CFB*. These age-related increases in gene expression as demonstrated on microarray and qPCR analyses (Supplementary Fig. 4) indicate increased expression and secretion of these proteins by aging retinal microglia. As C3 is a complement protein in the alternative pathway and CFB positively regulates complement activation in this pathway, this data indicate that 1) retinal microglia may regulate complement activation locally in the retina and that 2) with aging, the regulatory balance favors complement activation. We performed immunohistochemical analyses on the young adult (3–4 month old) and aged (20–24 month old) retina for C3 and CFB. Within the inner retina, very low levels of C3 immunopositivity were detected in both young and aged animals with no detectable immunopositivity in retinal microglia (data not shown). However, in the subretinal space, while minimal C3 immunopositivity was detectable in the young adult retina, immunopositivity within subretinal microglia was clearly present in cell somata and processes (Fig. 5A). Immunopositivity for CFB was similarly absent within the inner layers of the retina of both ages (data not shown). In the outer retina, CFB staining was weak and sparse in the subretinal microglia of young animals, but present prominently in subretinal microglia of aged animals (Fig. 5B). Immunopositivity for iC3b, formed from complement activation and C3 cleavage, was largely absent in the subretinal space in the young adult retina but prominently deposited on the apical surface of the RPE layer in the aged retina, suggesting an age-related activation of the complement system in the subretinal space resulting from changes in microglial complement regulation (Fig. 5C). The age-dependent changes in the extents of immunopositivity of these complement proteins within the subretinal space were quantitated and shown in Fig. 5D. Subretinal microglia in the aged animals demonstrated larger somata sizes (Fig. 5E) and less ramified morphologies (Fig. 5F).

4. Discussion

4.1 Age-related changes in gene expression relating to constitutive microglial support

In the current study, we carefully isolated microglial cells from the retina of different ages to examine age-related microglial gene expression across the entire span of adult aging. Gene ontology (GO) analysis showed that retinal microglia demonstrate age-related changes in the expression of genes regulating various aspects of mRNA and DNA metabolism, including the regulation of transcription and DNA binding (Table 1). Aging has been previously associated with altered rates of transcript and protein turnover in various cell types (Brewer 2002), including immune cells such as T cells (Cao *et al.* 2010). A similarly altered mRNA/

DNA metabolism appears to be present in aging microglia. We found that genes coding for small GTPases regulatory proteins (*Arhgap21, Arhgap22, Arhgef12, Cdc42se2, Farp2, Srgap3*) which regulate cellular morphology and cell migration in multiple cell types (Etienne-Manneville & Hall 2002), including microglia (Yan *et al.* 2012), showed age-related changes. Genes coding for proteins that influence actin and microtubule organization that underlie dynamic process outgrowth and motility were differentially expressed with aging, including *formin1* (Chesarone *et al.* 2010), *formin binding protein 1-like* (Lee *et al.* 2010), and *Rab13* (Sakane *et al.* 2010). These changes may underlie age-related decreases in microglial ramification and dynamic behavior (Damani *et al.* 2011), which may in turn influence the regulation of synapses (Wake *et al.* 2009; Schafer *et al.* 2012a) and neuronal activity (Li *et al.* 2012), as well as neuroprotective functions (Vinet *et al.* 2012). Canonical pathway analyses highlighted changes in the neurotrophin/TRK signaling pathway (Fig. 2) which has been related to the supportive influence of microglia on neurons and macroglia (Harada *et al.* 2002). *Neurturin*, a neutrophic factor required for normal retinal neuronal structure and function (Brantley *et al.* 2008), was down-regulated with aging. Also, Egr1, a microglia-expressed transcription factor (Langmann *et al.* 2009) implicated in neuroprotective immune mechanisms (Bakalash *et al.* 2011; Sharma *et al.* 2012), was found to decrease monotonically with age. Taken together, altered expressions of genes involved in mRNA/DNA metabolism, cellular motility, and neurotrophin signaling suggest molecular mechanisms related to how microglial support functions decline during aging (Streit & Xue 2009).

4.2 Age-related changes in gene expression relating to altered microglial inflammatory responses

The “inflamm-aging hypothesis” (Franceschi *et al.* 2000) has proposed that aging changes in microglia drive dysregulated inflammatory responses to stimuli (i.e. priming) (Dilger & Johnson 2008), which result in an increased risk of neurodegeneration in aged animals (Mrak & Griffin 2005). In aged brains, increased microglial activation is evidenced by a greater basal expression of activation markers (Perry *et al.* 1993; Sheng *et al.* 1998), elevated basal cytokine profiles (Ye & Johnson 1999; Sierra *et al.* 2007; Njie *et al.* 2012), and more severe neurodegeneration following stimulation or injury (Sugama *et al.* 2003; Godbout *et al.* 2005; Sandhir *et al.* 2008; Wasserman *et al.* 2008). In the aged retina, microglia demonstrate dysfunctional responses to injury and injury signals (e.g. ATP) which are slower to initiate but also more prolonged and less reversible (Damani *et al.* 2011). These phenomena indicate that the regulation of the onset and resolution of microglial activation are affected by aging. Using gene ontogeny (GO) enrichment analysis, we detected among aging-related genes a significant representation by molecular pathways that regulate microglial activation, including those regulating I-κB kinase and NF-κB transcription factor activity, MAPK activity and ERK1/2 signaling (O’Neill & Kaltschmidt 1997; Koistinaho & Koistinaho 2002; Kim *et al.* 2004). Canonical pathway analyses highlighted the following inflammatory signaling pathways: (1) signaling involving IL-17, a cytokine demonstrated to increase inflammatory factor production in microglia *in vitro* (Kawanokuchi *et al.* 2008) and in animal stroke models *in vivo* (Lv *et al.* 2011), (2) ceramide signaling, (3) IL1 signaling, (4) nitric oxide signaling, and (5) lipopolysaccharide-stimulated MAPK signaling. Interestingly, network analysis on age-regulated microglial genes also identified the following genes as “hubs” for multiple direct interactions: (1) Jun and Fos AP-1 proteins, which have been found to be activated in microglia in pro-inflammatory, neurodegenerative contexts (Herdegen & Waetzig 2001), and (2) Egr-1, a regulator of inflammatory gene expression in microglia (Friedle *et al.* 2011) that is expressed in the retina in injury and disease (Brand *et al.* 2005; Langmann *et al.* 2009; Sharma *et al.* 2012). However, the relationship between these gene expression changes and the eventual activation state of microglia is likely complex and involve altered balances in multiple parallel and interacting

regulatory pathways. For example, the expression of *MyD88*, a gene encoding a key adaptor protein in the activation of NF-κB via TLR receptors, was increased with advancing age. On the other hand, other genes associated with the transcription of pro-inflammatory genes, such as c-Jun and c-Fos of the AP-1 transcription factor complex, were decreased with advancing age. However, the combined consequences of these altered balances also do not appear to result in a strong polarization to a M1- or M2-like state as different individual M1 and M2 markers were found to both increase and decrease with advancing age without a clear polarization towards one state.

We found a significant representation of genes involved in lipid biosynthesis among aging-related genes (Table 1), including those implicated in immune cell regulation. *Ch25H,* the gene for the enzyme that catalyzes the formation of 25-hydroxycholesterol (25Ch) from cholesterol, was upregulated with aging. In macrophages, Ch25 was found to regulate survival (Joseph *et al.* 2004; Diczfalusy *et al.* 2009), cytokine expression (Joseph *et al.* 2003), and signaling to the adaptive immune system (Bauman *et al.* 2009). *Pla2g5,* which codes for a secreted phospholipase A_2 that regulates the synthesis of inflammatory mediators (arachidonic acid, prostaglandins, leukotrienes, and platelet-activating factor) (Murakami *et al.* 1997), the mobilization of macrophages (Ruiperez *et al.* 2009)and leucocytes (Lapointe *et al.* 2010), and microglial inflammatory responses (Yang *et al.* 2009) was also increased in expression with aging. Genes involved in the production of neurosteroids which control the magnitude and duration of microglial activation (Sierra *et al.* 2008; Gottfried-Blackmore *et al.* 2010; Saijo *et al.* 2011) were also altered in expression. A down-regulated gene, *Hsd17b1*, codes for 11β-hydroxysteroid dehydrogenase type 1, which activates the glucocorticoid cortisone (Seckl & Walker 2001) and induces the production of steroid hormone 5-androsten-3β,17β-diol (ADIOL) (Moeller & Adamski 2009), a key factor in limiting microglial activation (Gottfried-Blackmore *et al.* 2010; Saijo *et al.* 2011). As a result, age-related changes in these lipid biosynthetic pathways may underlie altered activation responses in aged microglia.

Other studies have previously examined the effect of aging on the expression of inflammatory genes on the level of the entire retina. One study found that inflammatory chemokines (e.g. CCL2, CCL12) and cytokines (e.g. TNF-α) (Chen *et al.* 2010) were up-regulated with aging, while another failed to find similar changes (Chen *et al.* 2008a). While we found similar aging trends between microglial-specific and whole retina transcripts for some genes (e.g. Egr1, C3), we discovered opposite trends in other genes (e.g. TNF-α). CXCL13, a chemokine associated with B-cell signaling (Lalor & Segal 2010) and a biomarker of neuroinflammatory conditions (Alvarez *et al.* 2013), was markedly up-regulated with aging in microglia-specific transcripts, a trend not detected in previous whole-retina analyses. These disparities may arise from different molecular aging changes in microglia versus other retinal cell types, a difference that is likely to be apparent given the relative scarcity of microglia among all retinal cells. How these specific changes result in altered interactions between senescent microglia and other retinal cells will be a focus for our future functional studies.

4.3 Age-related changes in gene expression relating to disease susceptibility

We related the results of our current analyses to what is currently known about the physiology and genetic susceptibility to age-related retinal disease. Age-related macular degeneration (AMD), the largest cause of blindness in the Western hemisphere (Resnikoff *et al.* 2004), demonstrates a strong age-related prevalence (Friedman *et al.* 2004a) and a suggestive relationship to microglial senescence (Ma *et al.* 2013). In this study, we discovered several age-related microglial genes that have also been previously associated with AMD pathobiology. Canonical pathways involved in VEGF ligand-receptor signaling and TSP-1 signaling (Table 2, Fig. 4A, B), which have been implicated in general

angiogenesis (de Fraipont *et al.* 2001; Chung & Ferrara 2011)and in ocular angiogenesis (Witmer *et al.* 2003; Hiscott *et al.* 2006), were highlighted, underscoring a connection between aging microglial changes and neovascular AMD disease. We also found that mRNA expression of VEGF and VEGFR2 were significantly increased during retinal microglial aging, possibly reflecting an increased ability of aged retinal microglia to promote angiogenesis (Fantin *et al.* 2010; Fischer *et al.* 2011), contributing in some part to the increased risk of neovascular AMD with age.

We also examined how the results of the current analysis relate to known genetic susceptibilities to AMD, in particular to genes in the complement pathway (Swaroop *et al.* 2009). Given that elevated levels of complement and complement activation products colocalize with AMD lesions (Mullins *et al.* 2000; Hageman *et al.* 2005; Lommatzsch *et al.* 2008), risk polymorphisms in complement genes likely result in increased complement activation, driving aspects of disease progression (Scholl *et al.* 2008; Montes *et al.* 2009; Hecker *et al.* 2010). However, how complement and complement activated becomes dysregulated in the aged retina is unclear. We and others have previously demonstrated that retina microglia are able to synthesize complement and complement regulatory proteins, and may indeed regulate complement activation locally (Collier *et al.* 2011; Luo *et al.* 2011; Rutar *et al.* 2011; Ma *et al.* 2013). We had also shown that accumulation of an age-related ocular lipofuscin constituent A2E alters complement protein expression to favor increased complement activation (Ma *et al.* 2013). In the current analysis, we discovered that two central complement genes, C3 and CFB, exhibited a monotonic-to-age increase in retinal microglia. We confirmed these findings in immunohistochemical studies where increased labeling of both C3 and CFB were observed in the outer retina of aged mice that colocalized with subretinal microglia. The deposition of activated C3 breakdown product, iC3b, was increased in the subretinal space in the areas of microglial accumulation.

Previous microarray studies examining age-related changes in ocular tissues such as in the whole retina (Chen *et al.* 2008b) and in the RPE-choroid complex (Chen *et al.* 2008a), have also described in increases in the expression of C3 and CFB, as well as other complement proteins. However the cellular source for these increases was undemonstrated. Our data here indicate that aging retinal microglia indeed constitute a cellular source of increased complement proteins, and can drive increased complement activation in the outer retina in ways that contribute causally to AMD progression. Corroboratively, early stage up-regulation of complement has also been identified in the retinas of a mouse model of glaucoma, another age-related retinal disease (Howell *et al.* 2011). Given the presence of early microglial activation in this mouse model (Bosco *et al.* 2011), and that interventions that decrease microglial activation can alleviate glaucomatous change (Bosco *et al.* 2008; Bosco *et al.* 2012), it will be interesting in future studies to confirm whether microglia indeed constitute the cellular locus for complement gene dysregulation in glaucoma. If true, complement dysregulation in aging retinal microglia may constitute a common cellular mechanism driving multiple age-related neurodegenerative diseases in the retina.

In conclusion, the findings from the current analysis indicate a number of molecular mechanisms that underlie functional and pathogenic changes in microglial aging including changes in constitutive supportive microglial functions and altered regulation of microglial activation. Compellingly, the results also highlight complement genes that have previously been identified as conferring risk for the age-related retinal diseases of AMD and glaucoma. Future work will focus on validating these potential molecular mechanisms in regulating features of the senescent phenotype in microglia, with the goal of understanding and therapeutically addressing the various forms of age-related neurodegeneration in the retina.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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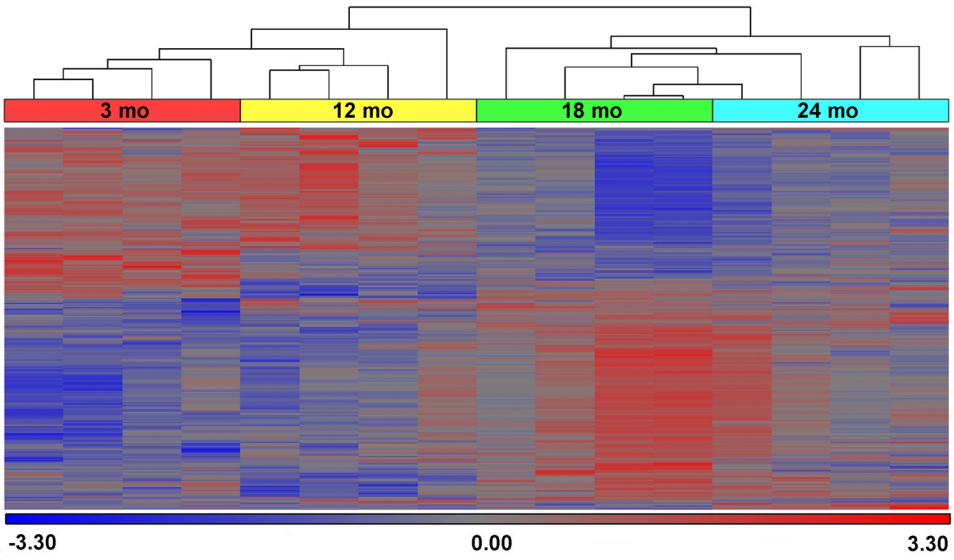

Figure 1. Hierarchical clustering heat map for 719 genes demonstrating age-related differential expression (fold-change ≥1.5x and uncorrected p-values <0.05)

Each column represents a biological replicate of a particular age (4 replicates per age group); each column row represents a single gene. Gene expression changes with respect to median changes are represented in direction and magnitude by the legend (*bottom*): red, up-regulated; blue, down-regulated; gray, unchanged.

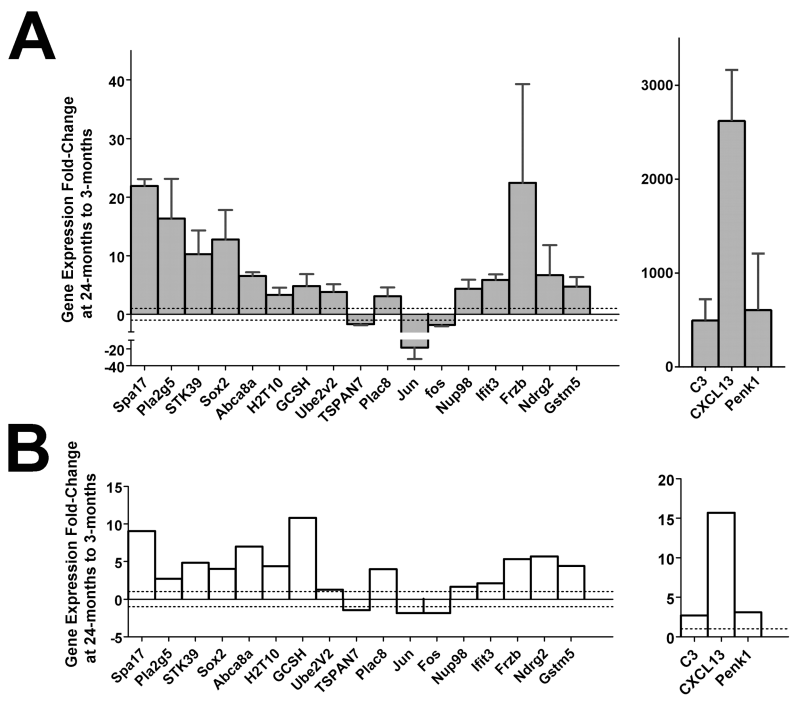

Figure 2. Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) validation of selected genes

Relative gene expression fold change from qRT-PCR from retinal microglia from 24-month old mice (3 -6 biological repeats were used for each experiment, with one sample replicate per biological replicate), normalized relative to those from 3-month old mice (**A**) is compared to the relative fold changes for the same genes predicted from microarray analysis (**B**). Error bars indicate ± SEM.

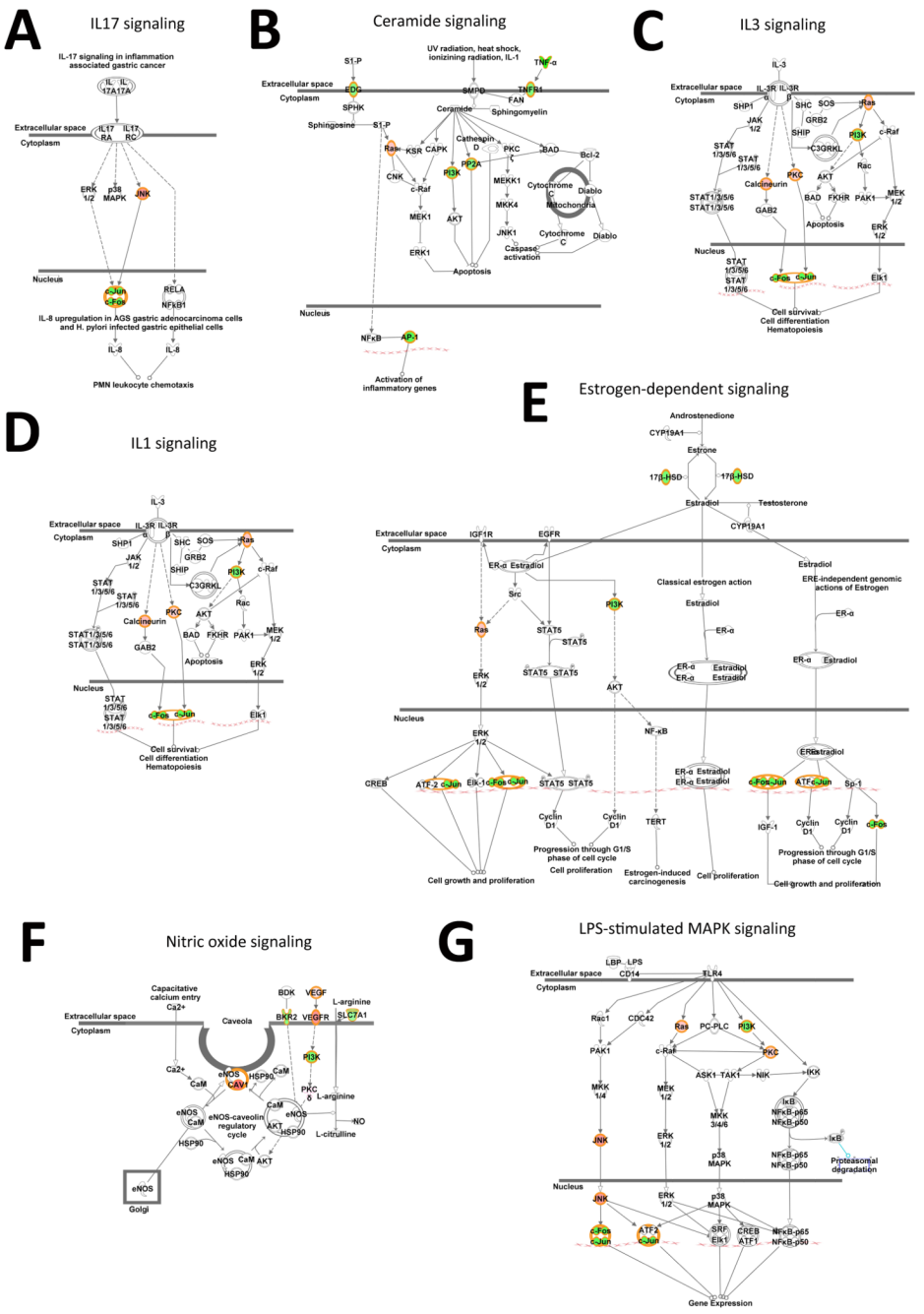

Figure 3. Age-associated gene expression changes in microglial signaling pathways involved with microglial immune function and activation as generated by Ingenuity Pathway analysis

Signaling pathways involved in the regulation of microglial immune activities were highlighted in the analyses of genes whose expression varied significantly as a function of age. Aging-related genes are indicated either in green (down-regulated) or in red (up-regulated) (for 3-month to 24-month comparisons). The most prominently represented pathways included (**A**) IL17 signaling, (**B**) ceramide signaling, **(C)** IL3 signaling, (**D**) IL1 signaling, (**E**) estrogen-dependent signaling, (**F**) nitric oxide signaling, and (**G**) lipopolysaccharide (LPS)-stimulated MAPK signaling.

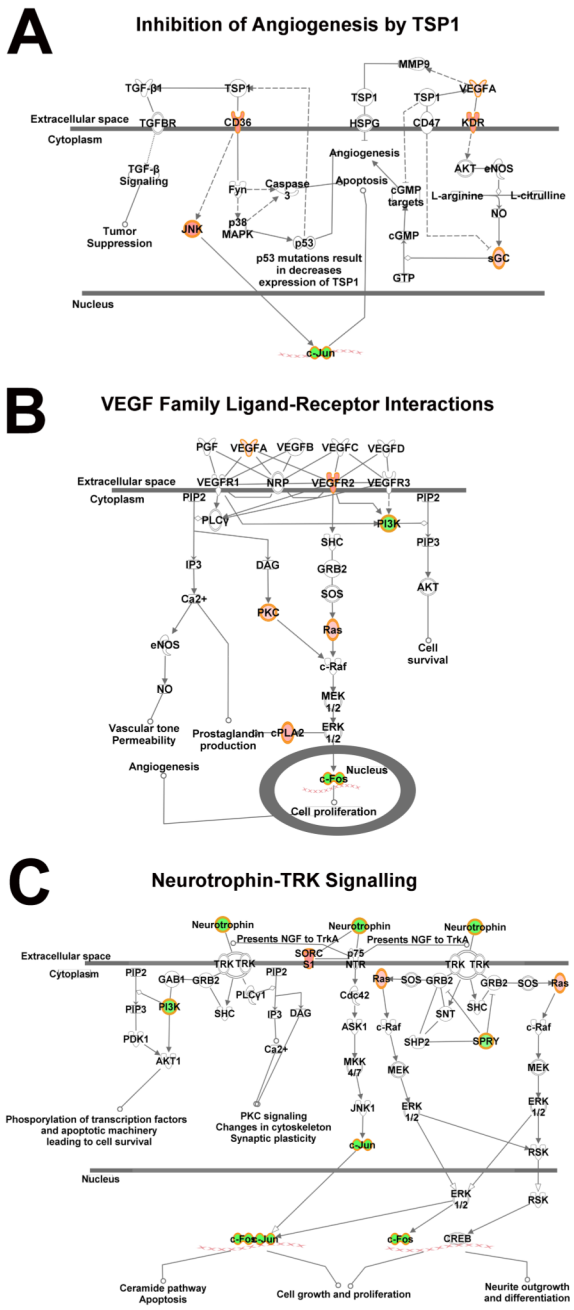

Figure 4. Age-associated gene expression changes in microglial signaling pathways involved with angiogensis and neurotrophic support as generated by Ingenuity Pathway analysis

(A–B) Two particular signaling pathways involved in angiogenesis were those involving (A) thrombospondin-1 (TSP1), and (B) vascular endothelial growth factor (VEGF). (**C**) Neurotrophic-TRK receptor signaling pathways were also highlighted by microglial genes demonstrating age-related change. Aging-related genes are indicated either in green (down-regulated) or in red (up-regulated) (for 3-month to 24-month comparisons).

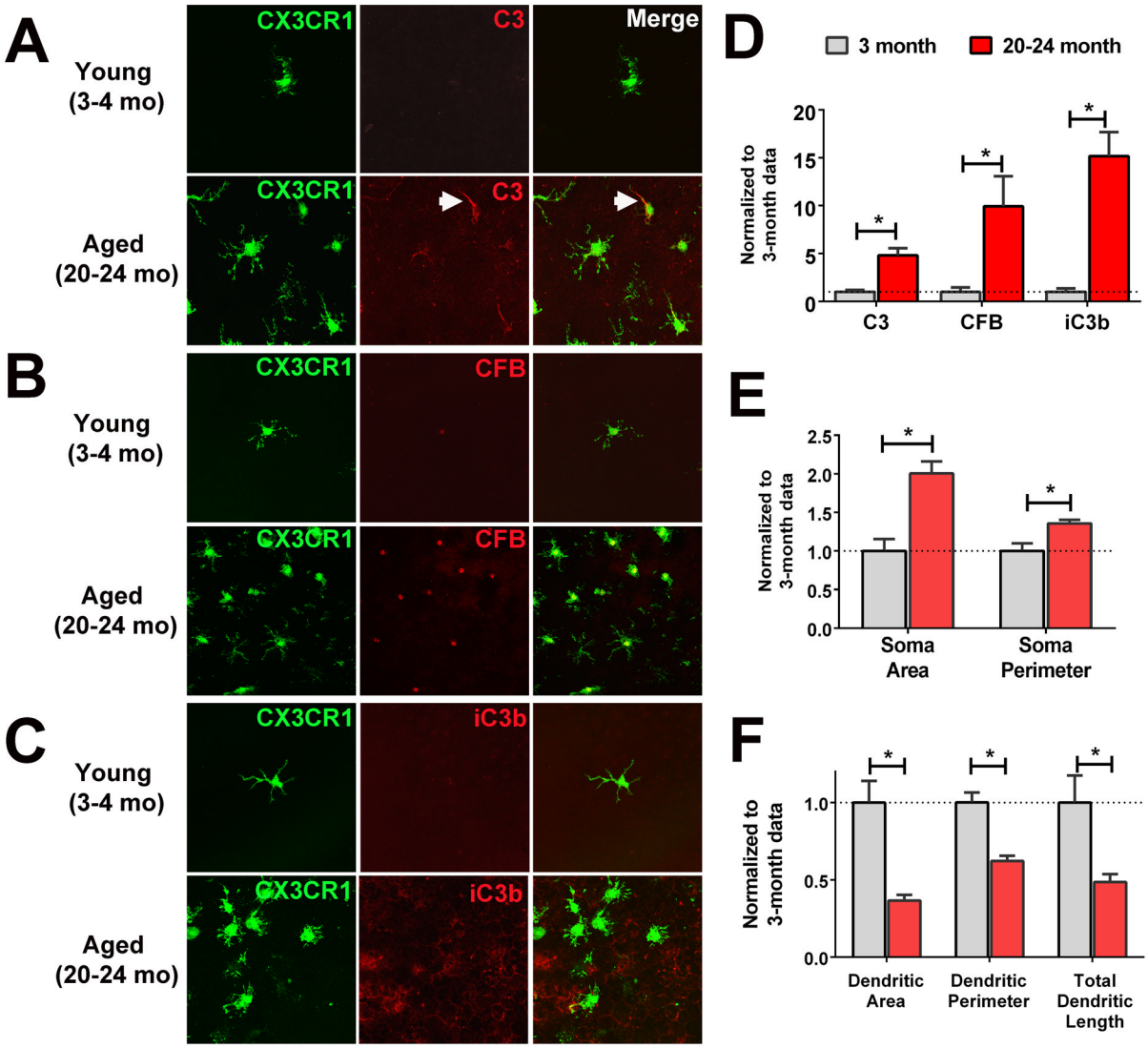

Figure 5. Age-related changes in the complement gene expression and complement activation in retinal microglia

Immunohistochemical staining of complement proteins, C3 (A) and CFB (B) in subretinal microglia, and complement activation product iC3b deposition in the subretinal space (C) of young (3–4 month old) and aged (20–24 month old) CX3CR1$^{+/gfp}$ transgenic mice. (**A**) Immunostaining for C3 (*red*) in subretinal microglia (*green*) demonstrated the absence of detectable immunopositivity in young mice. In aged mice, a substantial proportion of subretinal microglia showed immunopositivity, particularly in microglial processes (*arrow*). (**B**) Immunostaining for CFB (*red*) in subretinal microglia (*green*) demonstrated very weak immunopositivity in young mice but prominent staining of microglial somata in aged mice. (**C**) Immunopositivity for iC3b (*red*) was absent from the subretinal space of young mice but found distributed in a punctate manner in that of aged mice (*green*). (**D**) Quantification of the extent of immunopositivity of C3, CFB, and iC3b, demonstrating increased levels in the subretinal space in 20–24 month-old mice, relative to 3 month-old mice. (n = 6 representative 60x fields from 4–6 animals in each age group), (**E**) Morphological analysis of microglial soma sizes, demonstrating enlarged somata in terms of area and perimeter in 20–24 month-old mice, relative to 3 month-old mice. (**F**) Morphological analysis of

microglial dendritic territory sizes, demonstrating decreased ramification in terms of decreased areas, perimeter, and total dendritic lengths of individual microglia in 20–24 month-old mice, relative to 3 month-old mice (n = 9 representative cells from 4–6 animals in each age group). * indicates comparisons for which $p < 0.05$ (unpaired t-test with Welch's correction).

Table 1

Gene ontology (GO) enrichment analysis of 719 aging-associated genes in retinal microglia

		Cellular Process	Enrichment Score[1]
Metabolic process	lipid biosynthetic process	triglyceride metabolic process	8.21
		very long-chain fatty acid biosynthetic process	7.17
		platelet activating factor biosynthetic process	6.01
		steroid biosynthetic process	5.13
		isocitrate metabolic process	4.46
		fatty acid metabolic process	4.34
Biological Regulation	positive regulation of transcription, DNA- dependent	positive regulation of transcription from RNA polymerase II promoter	7.64
		negative regulation of DNA binding	7.34
		regulation of transcription, DNA-dependent	5.58
		regulation of transcription from RNA polymerase II promoter	3.22
	regulation of signal transduction	negative regulation of ERK1 and ERK2 cascade	6.71
		negative regulation of Rho protein signal transduction	6.34
		positive regulation of MAPKKK cascade by fibroblast growth factor receptor signaling pathway	4.46
		positive regulation of I-kappaB kinase/NF-kappaB cascade	3.7
		positive regulation of MAPKKK cascade	3.46
	Regulation of activity	inactivation of MAPK activity	6.48
		positive regulation of metalloenzyme activity	6.01
		positive regulation of protein serine/threonine kinase activity	4.78
		positive regulation of NF-kappaB transcription factor activity	3.23
	regulation of phosphorylation	positive regulation of peptidyl-serine phosphorylation	6.96
		negative regulation of peptidyl-serine phosphorylation	3.44
	regulation of homeostatic process	positive regulation of release of sequestered calcium ion into cytosol	9.97
	negative regulation of cellular process	negative regulation of cell proliferation	6.06
		negative regulation of cell growth	5.75
	regulation of localization	positive regulation of potassium ion transport	6.72
	regulation of cellular component organization	positive regulation of endocytosis	5.44
		positive regulation of protein complex assembly	3.44
Transport	organic substance transport	L-glutamate import	7.71
		D-aspartate import	6.01
		L-glutamate transport	5.7
		L-amino acid transport	4.26
		dicarboxylic acid transport	4.14
	intracellular protein transport	protein targeting	4.94

	Cellular Process	Enrichment Score[1]
secretion by cell	protein import into nucleus, translocation	4.26
	calcium ion-dependent exocytosis	5.44
	exocytosis	3.17
Immune system process	microglial cell activation	5.34
Locomotion	cell migration	6.01
Cellular component organization or biogenesis	histone ubiquitination	6.72

[1] Log transformation of the p-value calculated usinga chi-square test comparing the proportion of genes in the list showing differential expression in a given GO group to the proportion of all genes in the same GO group.

Table 2

Canonical pathway analysis of 719 genes differentially expressed with aging in retinal microglia.

Canonical Pathways	Age-related genes	Ratio[1]	P-value
Pathways related to microglial immune function and regulation			
IL-17A Signaling	FOS, JUN, MAPK10, TNF	0.1600	0.010000
Ceramide Signaling	S1PR4, FOS, JUN, S1PR5, PIK3C2A, TNFRSF1A, PPM1L, PIK3R6, MRAS, PPP2R5E, TNF	0.1260	0.000071
IL-3 Signaling	FOS, PRKCI, J UN, PIK3C2A, CHP, PIK3R6, MRAS, PRKCE	0.1080	0.002951
IL-1 Signaling	GNB1, FOS, JUN, PRKAR2B, MYD88, GNAO1, MRAS, MAPK10, PRKAR2A, GNG3, ADCY7	0.1040	0.000288
Nitric Oxide Signaling	VEGFA, BDKRB2, PRKAR2B, GUCY1A3, PIK3C2A, KDR, PIK3R6, CAV1, PRKAR2A, SLC7A1	0.1000	0.000372
Estrogen-Dependent Breast Cancer Signaling	FOS, JUN, PIK3C2A, PIK3R6, MRAS, HSD17B12, HSD17B1	0.1000	0.004365
LPS-stimulated MAPK Signaling	FOS, PRKCI, JUN, PIK3C2A, PIK3R6, MRAS, MAPK10, PRKCE	0.0976	0.003548
Pathways related to angiogenesis			
Inhibition of Angiogenesis by TSP1	VEGFA, JUN, GUCY1A3, KDR, MAPK10, CD36	0.1540	0.000955
VEGF Family Ligand-Receptor Interactions	VEGFA, FOS, PRKCI, PIK3C2A, KDR, PLA2G5, PIK3R6, MRAS, PRKCE	0.1070	0.001175
Pathways related to trophic factors			
Neurotrophin/TRK Signaling	FOS, JUN, Klk1b1, PIK3C2A, SPRY2, PIK3R6, MRAS, SORCS1	0.1100	0.001698

[1] Number of genes in the list of age-related genes in a given canonical pathway divided by the total number of genes mapping to that pathway.