Sex and APOE Genotype Alter the Basal and Induced Inflammatory State of Primary Microglia from APOE Targeted Replacement Mice

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Abstract

Background: Sex and APOE4 genotype are significant risk factors for Alzheimer's disease (AD), with females demonstrating increased risk that is modulated by APOE genotype. However, the mechanism(s) responsible for this interaction are still a matter of debate. Neuroinflammation represents a plausible mechanism for this interaction, but most experimental studies have not incorporated both sex and APOE genotype. Here, we sought to assess the response of sex-specific APOE3 and APOE4 primary microglia (PMG) in comparison to traditional mixed sex cultures.

Methods: Mixed sex and sex-specific PMG were generated from neonatal targeted replacement APOE3 and APOE4 pups. Purity over 98% was consistently obtained for cultures and the presence or absence of the *Sry* (Sex-determining region Y) gene by qPCR was used to determine male-specific microglia. PMG were treated with 10 ng/mL or a combination of lipopolysaccharide (LPS) and interferon-gamma (IFN γ). Pro- and anti-inflammatory cytokine profiles were assessed by quantitative RT-PCR and multiplex MSD assays.

Results: Mixed sex APOE4 PMG were found to have higher basal mRNA expression of pro-inflammatory cytokines including TNF α , and iNOS compared to APOE3 PMG which was accompanied by increased levels of secreted TNF α , but not nitric oxide. In sex-specific cultures, basal expression of IL1 β , TNF α , IL6, and iNOS was 2-3 fold higher in APOE4 female PMG compared to APOE4 males, with both being higher compared to APOE3 cells. Similar results were found for protein levels of these cytokines. Following an inflammatory stimulus, the expression of pro-inflammatory cytokines was upregulated in the order E4 female > E4 male > E3 female > E3 male in sex-specific cultures. Similar results were found with secreted cytokine levels.

Conclusions: These data indicate that the APOE4 genotype and female sex, together contribute to a greater inflammatory response in PMG isolated from targeted replacement APOE mice. These data are consistent with clinical data and sex-specific PMG may provide a platform for exploring mechanisms of genotype and sex differences in AD-related to neuroinflammation and neurodegeneration.

Background

Alzheimer's disease (AD) is a multifactorial progressive neurodegenerative disease primarily characterized by two core pathologies- amyloid- β and tau. Evidence over the last decade has suggested that the presence of a sustained immune response is a hallmark pathology in AD accompanied by activation of microglia, the resident immune cells of the brain (1). Whole genome sequencing and genome-wide association studies (GWAS) have identified more than 20 gene variants associated with AD (2, 3). Several of these genes, including *TREM2*, *CR1*, *MS4A*, *BIN1*, and *CD33*, are involved in regulating immune and inflammatory responses, and some are directly associated with the microglial cell type in the brain (4-8) Integrative systems-based analyses of gene regulatory networks in post mortem human late-onset AD (LOAD) brain specimens have identified immune-microglial molecular networks as most highly

associated with pathophysiology in LOAD (9). Identification of these genes and their association with AD strengthens the need to investigate the role and mechanism of the innate immune system in the pathophysiology of AD (10, 11).

Apolipoprotein E (APOE) is a polymorphic gene in humans coding for three common isoforms, APOE2, APOE3, and APOE4, that vary at positions 112 and 158 with allelic frequency in the population in the order E3> E4> E2 (12-14). APOE4 emerged as the strongest genetic risk factor for LOAD over the years through numerous clinical, pathological, epidemiological, and GWAS analyses (8, 15). A single inherited copy of the APOE ϵ 4 allele increases the risk of developing LOAD by ~3-4 fold, while two inherited copies increase the risk for the disease by 8-12-fold (16-18). Although APOE is primarily involved in lipid and cholesterol homeostasis, recent evidence from AD patients and mouse models of AD have identified genotype-specific effects on AD pathogenesis, including inflammation (19-24). While APOE is expressed in neuronal cells following injury (25) and is predominantly expressed in and secreted by astrocytes (26, 27), the presence of APOE has also been reported in microglia (28). The APOE4 genotype has been demonstrated to modulate neuroinflammation via microglial pathways in human AD patients as well as AD mouse models (29, 30). In mouse primary microglia, inflammatory processes induced by LPS alone or in combination with IFN γ , increased the secretion of pro-inflammatory cytokines and nitric oxide (NO) production, in an APOE genotype-dependent manner, with APOE4 promoting the strongest pro-inflammatory response (31-33). In the CNS, microglia-mediated neuroinflammatory responses are differentially modulated by the APOE genotype, and APOE4 is associated with increased microgliosis and neuroinflammation (29).

Females are at a greater risk for the development of AD. According to the 2021 Alzheimer's Association report, a staggering 3.5 million women (over 2/3 of patients) live with AD (13). Studies have indicated that women at 45 years of age have a 19.5% lifetime risk of developing AD compared to 10.3% in males, and these numbers increase with age to 21.1% and 11.6%, respectively (13, 34). Reports from longitudinal neuroimaging studies on the Alzheimer's disease neuroimaging initiative (ADNI) cohort indicate that females with AD accumulate higher levels of pathological proteins (A β and tau), have 1-1.5% faster atrophy rates in the hippocampus than males, and worsened cognitive abilities (35, 36). Accumulation of A β and tau are more pronounced in female APOE4 carriers than their male counterparts, suggesting a sex-by-genotype interaction of APOE4 in females (35, 37-39). Since the immune system plays a crucial role in the progression of AD, it is plausible to hypothesize that females have a greater predisposition to neuroinflammation in AD. Evidence from aging studies as well as AD models suggests that microglia play a key role in modulating inflammatory responses in females. Upregulation of microglia-specific immune genes, including *Aif1* (Allograft inflammatory factor 1/Iba1) and *Tyrobp* (tyrosine kinase binding protein/ DAP12), has been reported in older females compared to age-matched males (40). Sexual divergence has also been observed in AD patients and AD mouse models. Female microglia are more activated and differentially express microglial markers, change in density and morphology with thicker and longer processes, as well as demonstrate a switch in function from homeostatic to phagocytic (41-43).

The humanized targeted APOE mouse model (TR-APOE) was developed by introducing the human APOE gene to replace the murine APOE gene to study the APOE genotype effects on atherosclerosis (44). Although these mice lack the aggressive genetic mutations that drive early-onset AD, APOE genotype-specific effects have been observed in AD pathogenesis, including synaptic dysfunction (45), cholesterol (46), and lipid metabolism (47), with some studies highlighting an interaction in APOE genotype, age, and sex (48, 49). Differential effects of the APOE genotype on inflammation have also been explored in this model. TR-APOE mice that were chronically administered lipopolysaccharide (LPS) showed increased levels of pro-inflammatory cytokines TNF α and IL6 in both the serum and brain (50). Microglia derived from mixed-sex TR-APOE4 mice displayed a greater secretion of nitric oxide compared to the APOE3 genotype (51). Unfortunately, previous studies investigating APOE genotype-driven inflammatory differences in TR-APOE microglia have not assessed sex-specific variations (31, 51, 52). This is particularly important because females are at higher risk to develop AD (53, 54), female sex significantly affects the APOE4 risk for AD (38, 55), and sex has been indicated to modulate the microglial phenotype (56, 57).

Here, we sought to determine the sex and genotype-specific effects of LPS or LPS + interferon-gamma (IFN γ) using traditional mixed-sex and sex-specific APOE3 and APOE4 primary microglia (PMG) isolated from the TR-APOE3 and APOE4 mice. Our findings reveal differences in the inflammatory profiles of both genotypes influenced by sex, a key variable not considered in previous studies. Furthermore, our results indicate that LPS or LPS + IFN γ induces inflammation that is affected by both APOE genotype and sex, with a greater inflammatory profile observed with the APOE4 genotype and female sex in primary microglia. Collectively, these data demonstrate that multiple factors contribute to susceptibility to neuroinflammation and provide insight into the role of sex and genotype in this susceptibility.

Methods

Animals

TR-APOE breeder mice on a C57BL/6 background, with the mouse APOE gene replaced by homozygous human APOE3 or APOE4 gene (44), were purchased from Taconic and bred in-house. Breeders were given food and water *ad libitum* and housed in a 12-hour light-dark cycle. Primary microglial cultures were obtained from postnatal TR-APOE3 and TR-APOE4 pups aged 0-1 days. All procedures were conducted in accordance with the National Institutes of Health's Guide for Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of Florida International University.

Reagents

All cell culture reagents were purchased from Invitrogen (Waltham, MA), with the exception of charcoal-stripped fetal bovine serum (Cat. No. F6765) purchased from Sigma-Aldrich (St. Louis, MO). Lipopolysaccharide (LPS; *Escherichia coli* O127:B8, Cat. No. L4516) was purchased from Sigma-Aldrich, and interferon-gamma (IFN γ ; Cat. No. 485-MI-100) was purchased from R&D Systems (Minneapolis, MN).

Primary mouse microglia (PMG) isolation

Mixed-sex and sex-specific PMG cultures were isolated from postnatal 0-1 day old targeted replacement APOE3 or APOE4 mouse pups as previously described (58, 59). Briefly, whole brains were collected, trypsinized, thoroughly triturated, and passed through a 70 µm strainer. This mixed glial culture was maintained in flasks for 15 days in Dulbecco's Modified Eagle's Medium/F12 (DMEM/F12) supplemented with 10% charcoal-stripped heat-inactivated serum (HI-FBS), 2mM L-glutamine, 1mM sodium pyruvate, 100 µM non-essential amino acids, 1% Penicillin/Streptomycin and 2.5 µg/mL Plasmocin™. Charcoal-stripped fetal bovine serum was used to ensure no exogenous source of hormones interfered with the sex-specific results. Full media changes were done every 5-6 days with this growth medium. After 15 days, PMG were separated from other cell types by CD11b immunomagnetic positive selection kit (Cat. No. 18970, STEMCELL Technologies, Vancouver, BC, Canada) and cultured in the growth medium.

Following isolation, cell purity for PMG was assessed by imaging 3-4 random fields per well in a 96-well plate using the 10X objective on the Keyence BZ-X810 imaging instrument. Cells positive for Iba1 (microglia) or GFAP (astrocytes) were counted against the total number of cells (positive for DAPI) per field using the BZ-X800 Analyzer software, and the average was determined as percent purity. Cultures containing greater than 98% purity were consistently obtained. Prior to treatments, cells were validated for sex specificity by examining the presence or absence of the sex-determining region-Y (*Sry*) gene by qPCR.

Treatments

For cytokine and media nitrite measurement, PMG were seeded at a density of 140,000 cells/mL in 96-well plates. For qPCR experiments, 500,000 cells/mL were seeded in 24-well plates. For each isolation (N), cells were seeded in two separate plates (n), with two to three replicates for each treatment group per plate. Treatments were performed in growth media supplemented with 2% charcoal-stripped HI-FBS. PMG were treated with vehicle control (growth media containing 2% charcoal-stripped HI-FBS), LPS (10 ng/mL), or a combination of LPS (10 ng/mL) + IFNγ (10 ng/mL).

Conditioned Media Collection

Primary microglia were treated with vehicle control, LPS or a combination of LPS + IFNγ for 24 hours, during which cytokines were allowed to accumulate. Following 24 hours, media was briefly spun down, supernatant collected and stored in aliquots at -80°C until further use.

Measurement of secreted cytokines

Secreted cytokines were measured in the conditioned media using a custom U-PLEX mouse panel for the following analytes, IL1b, IL6, TNFa, IFNγ, MCP1, MIP-1a, IL-4, and IL10 on the Mesoscale Discovery

(MSD) platform following the manufacturer's instructions. Plates were read on the MSD QuickPlex SQ 120 and analyzed using the Discovery Workbench software.

Measurement of media nitrite

Nitric oxide is a signaling molecule that plays a key role in the pathogenesis of neuroinflammation and neurodegeneration (60). Media nitrite is one of the two stable breakdown products of nitric oxide. Media nitrite was quantified using the Griess reagent system (Promega) as previously described (61). Following 24 hour treatment, media was collected, and nitrite levels were measured at an absorbance of 535 nm along with a nitrite standard reference curve per the manufacturer's protocol.

Quantitative RT-qPCR

PMG were treated with LPS in the presence or absence of IFN γ for 6 hours. RNA was isolated using TRIzol Reagent following the manufacturer's instructions. cDNA was synthesized using the All-in-One cDNA Synthesis Supermix (Bio-Rad, Hercules, CA). Subsequent PCR reactions were performed using ItaqTM Universal SYBR green (Bio-Rad, Hercules, CA) following the manufacturer's instructions and previously described (62). A list of genes and primer sequences used in qPCR reactions is presented in **Supplementary Table 1**. Gene expression was normalized to Rpl13a using the $2^{-\Delta\Delta CT}$ method using the threshold cycle (CT) value for the housekeeping gene Rpl13a and the respective gene of interest (63).

Immunocytochemistry

PMG were seeded in 96-well plates, fixed with 4% paraformaldehyde for 15 minutes, washed with PBS, and immunocytochemistry was performed as previously described (61). Cells were permeabilized with PBS + 0.5% Triton X-100 and blocked for 1 hour in 2% bovine serum albumin (BSA) solution at room temperature followed by overnight incubation in 2% BSA with the following primary antibodies, GFAP (1:1000, Cat. No. ab4674, Abcam, Cambridge, UK) and Iba1 (1:1000, Cat. No. 019-19741, Wako Chemicals, Richmond, VA) at 4°C. The following day, cells were washed with PBS and incubated with Alexa Fluor® secondary antibodies from appropriate species (1:1000, ThermoFisher Scientific, Waltham, MA) for 1 hour at room temperature. After washing with PBS, cells were incubated with 10 μ g/mL DAPI for 15 minutes, washed again, and visualized under the microscope. The staining specificity was confirmed by omitting the primary or secondary antibody. Cells were imaged using the Keyence BZ-X810 microscope.

Statistics

Statistical analysis was performed using Prism 7.04 (GraphPad Software, San Diego, CA). Basal differences were analyzed in mixed-sex and sex-specific cultures by unpaired Student's t-test and two-way

analysis of variance (ANOVA), respectively. For comparisons, including LPS and LPS + IFN γ treated groups, mixed-sex cultures were analyzed using two-way ANOVA, whereas sex-specific cultures were analyzed by three-way ANOVA. Tukey's post hoc test to compare multiple groups was conducted following ANOVA. Statistical significance was considered at $p < 0.05$. Heat maps were generated using Displayr software (Pyrmont, Australia).

Results

APOE4 genotype has an increased inflammatory response to LPS in mixed-sex microglia

To investigate the effect of the APOE genotype on inflammation, microglia (PMG) from whole neonatal mouse brains were cultured (**Figure 1**). We first examined basal inflammatory gene expression levels in non-stimulated APOE3 and APOE4 microglia (**Figure 2**). Analyses for basal gene expression were carried out on dCT values normalized to *Rpl13a* and compared by a two-tailed unpaired Student's t-test. Basal expression of pro-inflammatory factors increased by 2 fold for *Tnfa* ($p < 0.0001$), 1.5 fold for *Il1b* ($p = 0.0002$), 1.8 fold for *Nos2* ($p = 0.0005$), and 2.4 fold for *Mcp1* ($p < 0.0001$) in the APOE4 microglia compared to APOE3. Similarly, basal differences in the anti-inflammatory cytokines were assessed, and significant increases in the baseline expression of *Arg1* ($p < 0.0001$), *Mrc1* ($p < 0.0001$), *Igf1* ($p < 0.0001$), and *Ym1* ($p = 0.0002$) were observed with 2.2, 1.5, 1.6 and 1.7 fold higher expression, respectively, in the APOE4 microglia. These basal differences suggest that APOE4 PMG innately have increased baseline levels of pro and anti-inflammatory cytokine expression. Although these increases are moderate, they may contribute to increased activation of PMG post an inflammatory stimulus.

Following treatment of mixed-sex PMG with LPS (10 ng/mL) alone or in combination with IFN γ (10 mg/mL) for 6 hours, an increase in the mRNA expression of pro-inflammatory cytokines was observed in both APOE3 and APOE4 genotype compared to their respective controls (**Figure 3A**). Two-way-analysis of variance (ANOVA) indicated a significant genotype effect for pro-inflammatory *Il6* ($F_{1,24} = 43.12$, $p < 0.0001$), *Il1b* ($F_{1,24} = 154.4$, $p < 0.0001$), *Tnfa* ($F_{1,24} = 153$, $p < 0.0001$), *Nos2* ($F_{1,24} = 13.51$, $p = 0.0012$), *Ifng* ($F_{1,24} = 51.96$, $p < 0.0001$) and *Mcp1* ($F_{1,24} = 46.47$, $p < 0.0001$) mRNA levels, with a higher increase observed in the APOE4 microglia. After exposure to LPS alone, APOE4 microglia showed higher mRNA levels of pro-inflammatory cytokines compared to APOE3 microglia. Expression increased approximately 1.3 fold in *Mcp1* ($p = 0.002$), 1.2 fold in *Nos2* ($p = 0.05$), 1.3 fold in *Ifng* ($p = 0.04$), 1.5 fold in *Il6* ($p < 0.0001$), 1.7 fold in *Il1b* ($p < 0.0001$) and 3 fold in *Tnfa* ($p < 0.0001$) compared to APOE3 microglia treated with LPS. Similarly, after a combination of LPS and IFN γ treatment, APOE4 microglia showed an approximate 1.3 fold increase in *Mcp1* ($p = 0.0001$), a 1.2 fold increase in *Nos2* ($p = 0.0085$) and *Il6* ($p = 0.0002$), a 1.5 fold increase in *Ifng* ($p < 0.0001$), and a 1.6 fold increase in *Il1b* ($p < 0.0001$) and *Tnfa* ($p < 0.0001$).

For anti-inflammatory cytokines analyzed after exposure to LPS or LPS + IFN γ , a significant genotype effect was observed for *Il10* ($F_{1,23} = 765.5$, $p < 0.0001$), *Arg1* ($F_{1,24} = 769.5$, $p < 0.0001$), *Mrc1* ($F_{1,24} = 190.8$, $p < 0.0001$), *Ym1* ($F_{1,24} = 1138$, $p < 0.0001$) and *Igf1* ($F_{1,24} = 582.6$, $p < 0.0001$) (**Figure 3B**). After exposure to

LPS alone, a 3.2 fold increase in *Il10* ($p<0.0001$) was observed in the APOE4 microglia compared to the APOE3 genotype. Smaller, yet significant, increases ranging from 1.5 to 2 fold were seen in APOE4 microglia in *Igf1* ($p<0.0001$), *Arg1* ($p<0.0001$), *Mrc1* ($p=0.004$), and *Ym1* ($p<0.0001$) compared to APOE3 microglia. However, a significant decrease in mRNA levels of *Fizz1* ($p=0.04$) was observed in the APOE4 genotype after LPS treatment, whereas no statistical difference was observed for *Tgfb* ($p=0.07$). In combination with IFN γ , LPS increased the expression of *Il10* by 2.5 fold ($p<0.0001$) in APOE4 microglia. Additionally, *Arg1* ($p<0.0001$) and *Mrc1* ($p=0.0002$) expression increased 1.5 fold whereas *Ym1* ($p<0.0001$) and *Igf1* ($p<0.0001$) mRNA levels increased 2 fold in APOE4 microglia. Similar to the LPS treatment, expression of *Fizz1* decreased ($p=0.0001$), and no change was observed in *Tgfb* ($p=0.22$). These data are summarized as a heat map in **Figure 3C**. Together, these data suggest that the microglial response to inflammation is influenced, at least in part, by the APOE genotype.

APOE4 genotype elevates secretion of inflammatory cytokine in mixed-sex microglial cultures

To explore APOE genotype-specific production of inflammatory mediators, microglia were stimulated with LPS for 24 hours in the presence or absence of IFN γ and the media collected. Media nitrite levels were significantly higher in the APOE4 microglia compared to APOE3 microglia by two-way ANOVA ($F_{1,32} = 61.85$, $p<0.0001$) following LPS treatment alone ($p<0.0001$) and in combination with IFN γ ($p<0.0001$) (**Figure 4**).

In addition to NO, microglia secrete inflammatory factors as a response to an inflammatory stimulus. To assess whether the APOE4 microglia released higher levels of cytokines, media collected after treatments were analyzed for both pro-inflammatory (IL6, TNFa, IL1b, IFN γ , MCP1, and MIP1a) and anti-inflammatory (IL10 and IL4) cytokines on the MSD platform. Basal secretion of IL1b, IL6, IFN γ , TNFa, MCP1, MIP1a, IL10, and IL4 was higher in APOE4 microglia by 3.5, 2.6, 3.2, 3.3, 3.9, 1.3, 1.7, and 3.5-fold respectively (**Figure 4B**). An increase in the secretion of pro and anti-inflammatory cytokines was detected after treatment with LPS and LPS + IFN γ in both APOE3 and APOE4 microglia (**Figure 4C**). However, these treatments induced significantly higher secretion of these cytokines from APOE4 microglia compared to APOE3. LPS treatment on the APOE4 microglia increased pro-inflammatory IL1b by 1.5 fold ($p=0.02$), IL6 by 2.3 fold ($p<0.0001$), TNFa by 4 fold ($p<0.0001$), IFN γ by 1.9 fold ($p<0.0001$), MCP1 by 1.2 fold ($p<0.0001$) and MIP1a by 2 fold ($p<0.0001$) and anti-inflammatory IL10 and IL4 by 3 fold ($p<0.0001$) and 1.7 fold ($p<0.0001$) respectively. Additionally, a combination of LPS and IFN γ increased pro-inflammatory IL1b by 2.6 fold ($p<0.0001$), IL6 by 1.4 fold ($p<0.0001$), TNFa by 2.4 fold ($p<0.0001$), IFN γ by 1.5 fold ($p<0.0001$), MCP1 by 1.2 fold ($p<0.0001$) and anti-inflammatory IL4 by 1.5 fold ($p<0.0001$). While there was a 1.6 fold increase in MIP1a and 1.3 fold increase in IL-10 following treatment with LPS + IFN γ , these data were not statistically significant by two-way ANOVA.

Basal inflammatory gene expression is driven by APOE genotype and sex

The impact of sex on AD pathology and inflammation is an important factor since the trajectories for progression of pathology in males and females are distinct. To evaluate the contribution of APOE genotype and sex in PMG to inflammatory stimulus, sex-specific primary cultures were generated from TR-APOE3 and APOE4 mouse pups. Percent purity of microglia in cultures was assessed (**Figure 1C and 1D**), and cells were validated for sex specificity by examining the presence or absence of the sex-determining region-Y (*Sry*) gene by qPCR (**Figure 1E**). Gene expression in non-stimulated male and female APOE3, and APOE4 microglia was measured following 6 hours to evaluate basal responses between genotypes and sexes (**Figure 5**). Results were normalized to the APOE3 male group, and basal mRNA levels were compared by two-way ANOVA with Tukey's posthoc testing. Differential responses in sex-specific microglia from APOE4 mice were observed with an increase in female *Il1b*, *Tnfa*, *Il6*, *Nos2* by 2-3 fold, and *Mcp1* by 1.7 fold compared to male microglia (**Figure 5A**). Furthermore, a significant interaction between the APOE genotype and sex was detected for *Il1b* ($F_{1,12} = 144.9$, $p < 0.0001$), *Tnfa* ($F_{1,12} = 532$, $p < 0.0001$), *Il6* ($F_{1,12} = 141.8$, $p < 0.0001$), *Nos2* ($F_{1,12} = 135$, $p < 0.0001$) and *Mcp1* ($F_{1,12} = 40.44$, $p < 0.0001$). Female microglia derived from APOE4 mice showed a higher gene expression in *Il1b* by 5.2 fold, in *Tnfa* and *Nos2* by 3 fold, in *Il6* by 3.8 fold, and 2 fold in *Mcp1* compared to APOE3 females. This suggests that in addition to the APOE genotype, sex also plays a key role in modulating inflammation.

The basal gene expression profiles for homeostatic and anti-inflammatory genes were also investigated in APOE3 and APOE4 male and female microglial cultures (**Figure 5B**). A significant interaction between genotype and sex was detected for *Il10* ($F_{1,12} = 9.54$, $p = 0.009$), *Mrc1* ($F_{1,12} = 93.25$, $p < 0.0001$), *Igf1* ($F_{1,12} = 87.72$, $p < 0.0001$), *Tgfb* ($F_{1,12} = 45.01$, $p < 0.0001$) and *Fizz1* ($F_{1,12} = 13.92$, $p = 0.0029$). Basal gene expression for female APOE4 was upregulated compared to APOE4 male for *Il10* ($p = 0.006$), *Arg1* ($p = 0.006$), *Mrc1* ($p < 0.0001$), and *Tgfb* ($p = 0.0015$), and no change was observed in *Fizz1*, *Igf1*, and *Ym1*. Contrastingly, gene expression in APOE3 females was downregulated for *Mrc1* ($p = 0.0055$), *Igf1* ($p < 0.0001$), and *Tgfb* ($p = 0.004$) compared to APOE3 males, whereas no difference was observed for *Il10* and *Ym1*. These basal differences between sexes of each genotype indicate that distinct responses to inflammation may be observed in part due to varying basal levels of homeostatic and anti-inflammatory gene expression.

Sex differences in inflammatory gene expression of immune stimulated TR-APOE3 and TR-APOE4 microglia following stimulus

Subsequently, the differences in gene expression to induced inflammation via LPS alone or a combination of LPS and IFN γ were investigated and analyzed using 3-way ANOVA with Tukey's posthoc testing. Pro-inflammatory cytokine mRNA expression in microglia was detected in the order E4 female > E4 male > E3 female > E3 male (**Figure 6A**). A significant interaction between treatment and sex, treatment and genotype, as well as treatment, genotype, and sex, was observed for most cytokines.

Following LPS treatment, sex-specific differences were observed in both APOE3 and APOE4 microglia. Pro-inflammatory cytokines increased 1 to 1.5 fold in APOE3 female microglia compared to male microglia, with a similar increase observed in the APOE4 microglia. Comparison between female cultures of APOE3 and APOE4 microglia revealed a 1.5 to 2-fold increase in APOE4 female pro-inflammatory mRNA levels. A similar pattern of fold increase was observed after treatment with LPS+ IFN γ (**Figure 6A**).

Following treatment with LPS or LPS + IFN γ , gene expression of homeostatic and anti-inflammatory genes was upregulated in the order E4 female > E4 male > E3 female > E3 male (**Figure 6B**). A significant interaction between treatment and sex, treatment and genotype, as well as treatment, genotype, and sex, was observed for a majority of cytokines. LPS treatment induced a 1.5 to 2 fold increase in female microglia cultures of each genotype compared to the male cultures of the corresponding genotype. Comparison between the female cultures of both genotypes indicated a 2 fold increase in *Il10* ($p<0.0001$), *Igf1* ($p<0.0001$), *Ym1* ($p=0.02$), and *Tgfb* ($p<0.0001$), and a 3 fold increase in *Arg1* ($p<0.0001$) in the APOE4 female microglia. Cultures treated with LPS + IFN γ showed similar results. These data are summarized as a heat map in **Figure 6C**. Together, these data indicate that several pro-inflammatory and anti-inflammatory genes are upregulated in female APOE4 microglia, and exposure to an inflammatory stimulus exacerbates the induction of these genes.

Female APOE4 microglial cultures have increased secretion of inflammatory cytokines

Nitric oxide levels were measured indirectly by quantifying levels of media nitrite in media collected from APOE3 and APOE4, male and female microglial cultures that were >98% pure. Data analysis using three-way ANOVA indicated a treatment effect on genotype ($F_{3,48} = 37.83$, $p<0.0001$) and sex ($F_{3,48} = 12.52$, $p<0.0001$). LPS treatment alone increased media nitrite levels significantly in APOE4 male and female microglial cultures compared to the respective vehicle control, whereas LPS + IFN γ increased media nitrite in both APOE3 and APOE4 male and female cultures (**Figure 7A**). Furthermore, female APOE4 microglia secreted 2.6 fold more media nitrite compared to APOE3 females and 1.7 fold more compared to APOE4 male cultures.

Accumulation of other inflammatory factors was also evaluated in the male and female cultures using the MSD multiplex assays (**Figure 7B and 7C**). Basal secretion of IL1b, IL6, TNFa, MCP1, MIP1a, and IL10 was higher in APOE4 microglia (**Figure 7B**). In addition, female cultures from both genotypes secreted approximately 1.5 to 2 fold higher levels of these cytokines, indicating sexual dimorphism in response to inflammation following treatment with LPS or LPS + IFN γ . A treatment by genotype by sex effect was observed for pro-inflammatory cytokines including IL6 ($F_{2,60} = 48.59$, $p<0.0001$), TNFa ($F_{2,60} = 163$, $p<0.0001$), MCP1 ($F_{2,60} = 11.97$, $p<0.0001$) and MIP1a ($F_{2,60} = 19.36$, $p<0.0001$) as well as anti-inflammatory IL10 ($F_{2,84} = 25.56$, $p<0.0001$) and IL4 ($F_{2,36} = 9.87$, $p=0.0004$). APOE4 female microglia had the highest accumulation of both pro and anti-inflammatory cytokines compared to APOE3 male and female as well as APOE4 male microglia (**Figure 7C**).

Discussion

Neuroinflammation is a concerted process in which microglia play a key role in regulating the immune response. Activation of the innate immune system, reflected by a milieu of pro and anti-inflammatory cytokines and chemokines, is a hallmark pathological feature of several neurodegenerative diseases, including AD (64). The transcriptional profile of microglia in AD shifts from a homeostatic phenotype to inflammatory modules such as disease-associated microglia (DAM) (65, 66), human Alzheimer's microglia (HAM) (67), or microglial neurodegenerative phenotype (MGnD) (30). Recent GWAS have identified more than 20 gene variants associated with AD, of which several genes including TREM2, CD33, and CLU are involved in regulating immune responses (2) and are directly associated with microglia (7). Interestingly, APOE has been shown to regulate some of these microglial transcriptional signatures as demonstrated in post mortem human brain studies, AD mouse models, and in vitro microglial cultures derived from both humans and mice (30, 65, 68, 69). A meta-analysis of several GWAS in AD has indicated APOE4 as the most significant genetic risk factor across populations influencing the prevalence and age-at-onset in an isoform-dependent manner (15, 16, 70).

In this study, we first evaluated the inflammatory response of mixed-sex microglia generated from both the TR-APOE3 and APOE4 mice. Consistent with prior literature, we observed an increase in the inflammatory profile of APOE4 microglia both basally and following stimulation with LPS or a combination of LPS and IFN γ . While APOE4 microglia have been implicated in exacerbating systemic as well as neuroinflammation, the genotype-dependent effects on neuroinflammation have been studied in mixed-sex microglial culture. To address this, we next investigated the differential inflammatory response of microglia based on APOE genotype and sex in TR-APOE mice. Across both sexes and genotypes, we observed an increase in inflammation post LPS treatment. This upregulation was found to be sex-specific for both genotypes, with females exhibiting greater inflammatory gene expression and cytokine secretion. Lastly, we report that sex and genotype together influence the microglial inflammatory profile with APOE4 female microglia showing elevated inflammatory cytokines and gene expression compared to APOE4 male as well as APOE3 male and female microglia.

APOE4 genotype has been associated with enhanced immune responses in humans, mouse models as well as in vitro cultures (68, 71, 72). Glial cultures consisting of both microglia and astrocytes generated from Sprague-Dawley rats produced higher levels of IL1 β after exposure to recombinant APOE4 (71). Although statistical significance was not reached, enhanced production of several cytokines and chemokines was seen in human whole blood of APOE4 carriers (72). Human clinical data from AD patients reported elevated plasma levels of the pro-inflammatory cytokines TNF α , IL6, and IL1 β in APOE4 carriers, carrying either one or two copies compared to that of APOE2 and APOE3 carriers (73, 74). In agreement with this, we observed basal elevation in the media levels and gene expression of pro-inflammatory cytokines, including in the non-stimulated APOE4 microglia compared to the APOE3 genotype. This suggests that APOE4 microglia inherently have an increased pro-inflammatory phenotype. A recent study by Lanfranco and colleagues in the TR-APOE mice showed no differences in basal TNF α mRNA across genotypes (52). In contrast, we observed a 1.5 to 2 fold increase in the basal TNF α mRNA

of APOE4 microglia compared to APOE3. A possible reason that could largely contribute to conflicting findings is the variability in the method used to generate primary microglia (CD11b targeted magnetic separation yielding >98% purity vs. the flask shaking method that yields 90-95% enriched microglial culture). Previous studies that evaluated the effect of inflammation on microglia in an APOE isoform-dependent manner saw similar genotype effects with high concentrations of LPS (100 – 500 ng/mL) (32, 52), although concentrations as low as 5 ng/mL have induced substantial inflammation (52). Furthermore, on investigating the cultures from both genotypes in a sex-specific manner, we found that both APOE3 and APOE4 females had a more pronounced inflammatory profile. Importantly, this is consistent with clinical data from healthy individuals carrying the APOE3 or APOE4 gene, APOE4 females show a higher increase in both pro-inflammatory cytokine release as well as gene expression (72, 75, 76).

Cultured mixed-sex microglia from TR-APOE mice have reported induction of media NO following treatment with IFN γ or IFN γ + Polyinosinic: Polycytidylic acid (PIC, a TLR3 ligand), with higher levels observed in the APOE4 cultures (51). Similarly, IFN γ alone or in combination with LPS increased media nitrite as well as NOS2 mRNA levels in APOE4 PMG (31). These effects were observed at a high (100 ng/mL) dose of LPS or at a later timepoint (31, 32). Our findings from mixed-sex cultures treated with LPS or LPS+IFN γ are consistent with the previously published reports, and we indeed observe increased NO levels and approximately 1.2 fold increase in NOS2 mRNA from APOE4 microglial cultures. Additionally, we also noted a basal increase in the NOS2 mRNA levels of APOE4 microglia. An increase in the pro-inflammatory cytokines such as TNF α and IL6 has also been reported in the supernatant of APOE4 microglia (31). We validated these results in the media from mixed-sex APOE4 cultures along with an increase in the gene expression of these cytokines.

We also identified novel sex differences in the response of microglia to inflammation. The observed sex differences are apparent in both APOE3, and APOE4 genotypes, with the greatest induction of inflammation, observed in APOE4 female microglia. While the specific mechanism elucidating the interaction between APOE4 genotype and female sex remains ambiguous, our findings are consistent with largely recognized studies in humans and TR-APOE mice indicating sex and APOE genotype differences in the inflammatory pathways (77-79). Sex-based differences in microglial response to inflammation has been reported, with studies indicating that females are relatively predisposed to a more robust inflammatory response (40, 57, 80). In addition, transcriptomic analyses in adult microglia have revealed significant sex differences that were at least partially independent of adult sex hormone exposure (40, 56). In order to reduce the effects of exogenous hormones in cultures, we supplemented the growth and treatment with charcoal-stripped FBS, suggesting that the effects we observed are predominantly the result of intrinsic sex differences.

Microglia have been implicated as potential modulators of sex differences and inflammatory profiles in neurodegenerative diseases, including AD (30, 57). Abundant evidence from previous human studies indicates sex differences stratified by APOE genotype in AD (35). While much of the female predisposition to AD has been reflected on longevity, mounting evidence supports that other factors, including inflammation, may also potentiate the risk in AD for females (43). Assessment of microglial

metabolism across AD patients and mouse models has revealed that female microglia are relatively activated and present increased inflammatory gene expression (42). We speculate that these sex-specific responses in AD likely involve, but are not limited to, the role of microglia.

Although the mechanism through which APOE isoforms differentially modulate neuroinflammation is unclear, the APOE protein has been demonstrated to exert immunomodulatory effects (50). APOE has been shown to reduce microglial activation and subsequent release of inflammatory mediators in an APOE genotype-specific manner (81-83). Recently Lanfranco and colleagues showed that microglia derived from TR-APOE4 mice had significantly lower levels of secreted and cellular APOE compared to the APOE2 genotype (52). While no difference was observed between APOE3 and APOE4 genotype, using highly purified microglial cultures (>99%) might provide additional insight into potential differences. They further found that stimulating the microglia with LPS increased secretion of APOE in the APOE2 and APOE3 microglia with no changes observed in the APOE4 microglia. Conversely, exposure to TNF α increased ApoE secretion in the APOE4 microglia, but secretion from APOE2 and APOE3 remained unchanged. Overall, these data indicate isoform-dependent regulation of APOE in microglia, which is further modified by different inflammatory insults that may exacerbate neuroinflammation. Studies assessing APOE levels in brain lysates of TR-APOE mice indicated reduced levels of the APOE protein in APOE4 mice compared to the APOE3 genotype (31). However, even after controlling for APOEE levels, higher pro-inflammatory responses were observed in the APOE4 mice suggesting that APOE4 may regulate immune response independent of APOE protein concentration (31).

Another potential mechanism by which APOE4 may result in an enhanced response to inflammatory stimulus is through various signaling pathways. LPS activates CD14/TLR4 coreceptors, leading to increased inflammation in either p38MAPK or NF- κ B dependent manner (32). Maezawa and colleagues demonstrated that the APOE4 microglia differentially regulates the p38 mitogen-activated protein kinase signaling pathway, subsequently resulting in increased cytokine secretion by APOE4 microglia compared to APOE3 microglia (32). A growing body of evidence has also shown that APOE4 enhances inflammation in the brain by modulating the NF- κ B signaling, which is a crucial transcription factor regulating immune gene expression (84, 85). Furthermore, the APOE4 genotype is linked to a partial loss of function, and its inefficacy to suppress an inflammatory response could be a plausible explanation for an increased inflammatory profile in the APOE4 genotype. Importantly, these mechanisms have not been explored in a sex-specific manner and are the basis of ongoing studies in our laboratory. Our findings add to the growing literature indicating the importance of including both APOE genotype and sex to study microglia-mediated inflammation in AD.

Although the current study was conducted in microglia from the whole brain, recent evidence has indicated that microglia possess regional transcriptional heterogeneity and region-specific functions (86, 87). Microglial genes from the cortex show upregulation in immune-activated microglial phenotype, whereas the hippocampal microglial gene cluster is highly involved in bioenergetic maintenance (86). Peripheral endotoxin-induced inflammation has previously been shown to alter the gene expression of cytokines and immune cell markers in a brain region-specific manner, with microglia, also demonstrating

region specificity in the inflammatory profile (88, 89). A recent comprehensive study showed APOE4 associated differential regional vulnerability utilizing transcriptomics analysis (90). Because the frontal cortex and hippocampus are the most vulnerable regions for developing AD pathology, investigating the region-specific activation of microglia could provide mechanistic insights. Additionally, we used neonatal mouse pups to generate primary microglial cultures. Evidence from the characterization of inflammation from neonatal and adult primary microglial shows a similar pattern of response to TLR agonists, including LPS. However, the magnitude of response differs, with adult primary microglia demonstrating a more robust response, suggesting that the data we report in neonatal microglia may be underestimating the effects observed in adults(91). Future studies will focus on profiling inflammation from adult/ aged animals, thus defining the *in vitro* modulation of genotype and sex by age differences as aging influences glial phenotype and molecular profile (92, 93).

Conclusions

Collectively, these data indicate that the APOE4 genotype and female sex together contribute to a greater inflammatory response in microglia isolated from targeted replacement APOE mice. Currently, there is a lack of comprehensive assessments of sex differences in microglia stratified by APOE genotype. While effects of inflammation have been studied independently in a sex-specific or an APOE isoform-dependent manner, very few studies have investigated how APOE genotype and sex together modulate the response to inflammation (78). These findings provide a compelling rationale for the inclusion of both male and female sexes to evaluate the role of inflammation in AD. To our knowledge, the results presented in this study are the first data in TR-APOE mice to show significant sex-specific effects after an inflammatory stimulus in cultured microglia. These data are consistent with clinical data, and sex-specific microglia may provide a platform for exploring mechanisms of genotype and sex differences in AD, particularly as they relate to neuroinflammation and neurodegeneration.

Abbreviations

AD: Alzheimer's Disease

GWAS: Genome-wide association studies

LOAD: Late-onset Alzheimer's Disease

APOE: Apolipoprotein

NO: Nitric oxide

ADNI: Alzheimer's disease neuroimaging initiative

TR-APOE: Targeted replacement – APOE

LPS: Lipopolysaccharide

IFNg: Interferon-gamma

PMG: Primary microglia

Declarations

Ethics approval and consent to participate

All mice were housed at Florida International University – Animal Care Facility. Procedures were conducted in accordance with the National Institutes of Health’s Guide for Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of Florida International University.

Consent for publication

Not Applicable

Availability of data and materials

All data generated or analyzed during this study are included in this published article. Further information/data is available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors’ contributions

IMW, AE, KT and JRR contributed to the conceptualization and design of the research; IMW and YH generated cultures and acquired data; JRR and IMW interpreted data; IMW drafted and revised the manuscript; KT and JRR provided revisions. All authors read and approved the final manuscript.

Acknowledgments

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Figures

Figure 1

Figure 1

Purity of mixed-sex and sex-specific microglial cultures

(A) Representative images for purity of mixed-sex microglial cultures. (B) Purity of mixed-sex microglial cultures was greater than 98%. (C) Representative images for purity of sex-specific microglial cultures. (D) Purity greater than 98% was consistently obtained for all sex-specific microglial cultures. Each data point

in figure B and D denotes an average percent of microglia from 3 to 4 images per well. (E) Sex-specificity of cultures was validated by checking for the presence or absence of sex-determining region-Y (*Sry*) by qPCR. Each data point represents the average mRNA expression from one isolation. Data are represented as mean $\pm$ standard deviation.

Figure 2

Figure 2

Basal pro and anti-inflammatory gene expression in mixed-sex microglia is APOE genotype dependent

(A) Basal pro-inflammatory and (B) anti-inflammatory gene expression was evaluated in non-stimulated mixed-sex microglia from APOE3 and APOE4 genotypes. Gene expression between genotypes was analyzed using unpaired Student's t-test (N=3 isolations, n=6 plates). Data are represented as mean $\pm$ standard deviation, ns = not statistically significant ($p>0.05$).

Figure 3

Comparison of gene expression between APOE3 and APOE4 genotypes

(A) Pro and (B) anti-inflammatory gene expression was evaluated in mixed-sex microglia from APOE3 and APOE4 genotypes following treatment with LPS (10 ng/mL) and LPS (10 ng/mL) + IFN γ (10 ng/mL) for 6 hours. Gene expression between genotypes was assessed by Two-way ANOVA followed by Tukey's multiple comparisons test (N=3 isolations, n=6 plates). Each data point represents average mRNA expression from one isolation. # denotes significant increase compared to respective genotype control (data not included in the graphs, $p < 0.05$). Data are represented as mean $\pm$ standard deviation. (C) Heat map comparing the mean expression of pro and anti-inflammatory genes in control, LPS and LPS + IFN γ treated APOE3 and APOE4 PMG. * and ^ denote significant ($p < 0.05$) increase and decrease, respectively, in APOE4 gene expression compared to APOE3.

Figure 4

Figure 4

APOE4 genotype exacerbates release of pro and anti-inflammatory cytokines

Cytokine levels were measured following treatment with LPS or LPS + IFN γ for 24 hours. (A) Average levels of nitrite ($\pm$ standard deviation) accumulated in the media were measured by GRIESS assay. Using

two-way ANOVA, significant treatment and genotype effects were observed. # denotes a significant increase compared to respective genotype control ($p<0.05$). (B) Basal levels of secreted cytokines were measured in non-stimulated microglia after 24 hours by MSD multiplex assay. Comparisons between genotypes were made using unpaired Student's t-test. (C) Cytokine levels in media were measured after treatment with LPS or a combination of LPS and IFN γ for 24 hours. Two-way ANOVA was used to assess cytokine release in mixed-sex microglia from APOE3 and APOE4 genotypes. (N=3-4 isolations, n=6-8 plates). Data are represented as mean $\pm$ standard deviation and each data point denotes the average of each experimental plate. # denotes significant increase compared to respective genotype control (data not included in the graphs, $p<0.05$). ns = not statistically significant ($p>0.05$).

Figure 5

Basal pro and anti-inflammatory gene expression in sex-specific microglia is dependent on both APOE genotype and sex

(A) Basal pro-inflammatory and (B) anti-inflammatory gene expression was evaluated in non-stimulated sex-specific microglia from APOE3 and APOE4 genotypes. Gene expression between genotypes and sexes was analyzed using a two-way ANOVA (N=3 isolations, n=6 plates). Data are represented as mean $\pm$ standard deviation, ns = not statistically significant ($p>0.05$).

Figure 6 ■ E3 Male ■ E3 Female ■ E4 Male ■ E4 Female

Figure 6

Gene expression in sex-specific microglia is dependent on both APOE genotype and sex

(A) Pro and (B) anti-inflammatory gene expression was evaluated in sex-specific microglia from APOE3 and APOE4 genotypes following LPS or LPS + IFNg treatment for 6 hours. Gene expression between genotypes and sexes was analyzed using a three-way ANOVA (N=3 isolations, n=6 plates) followed by Tukey's multiple comparisons test. Each data point represents average mRNA expression from one isolation. # denotes significant increase compared to respective genotype control (data not included in the graphs, $p < 0.05$). Data are represented as mean $\pm$ standard deviation, ns = not statistically significant ($p > 0.05$). (C) Heat map comparing the mean expression of pro and anti-inflammatory genes in sex-specific APOE3 and APOE4 PMG treated with LPS and LPS + IFNg. * and ^ denote significant ($p < 0.05$)

increase and decrease, respectively, in fold changes of gene expression compared to genotype-matched male PMG.

Figure 7

Increased secretion of inflammatory cytokines is dependent on both APOE genotype and sex

(A) Average nitrite levels accumulated in the media were measured by GRIESS assay. Three-way ANOVA followed by Tukey's multiple comparisons test revealed a significant treatment-by-genotype-by-sex effect. # denotes significant increase compared to respective genotype control ($p < 0.05$). (B) Basal levels of secreted cytokines were measured in non-stimulated microglia after 24 hours by MSD multiplex assay. Comparisons between sex and genotype were made using two-way ANOVA followed by Tukey's multiple comparisons test ($N = 3$ isolations, $n = 6$ plates). (C) Secretion of cytokines was measured post treatment with LPS or LPS + IFNg by a three-way ANOVA followed by Tukey's multiple comparisons test. # denotes

significant increase compared to respective control (data not included in the graphs, $p < 0.05$). Data are represented as mean $\pm$ standard deviation, ns = not statistically significant ($p > 0.05$).

Supplementary Files

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- [Supplementarytable1.xlsx](#)