# Apolipoprotein E4 expressed by microglia impairs microglial functions and enhances neurotoxicity

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# Abstract

**Background** Microglia, the major cell type that mediates active immune defence in the central nervous system (CNS), constantly survey the brain parenchyma through highly motile processes. Mounting evidence has implicated both beneficial and toxic roles of microglia when over-activated upon neuronal injury. Understanding the function of microglia in the brain may uncover the regulatory mechanisms for neuroinflammation and facilitate the development of a novel therapeutic strategy for Alzheimer's disease (AD). The ε4 allele of apolipoprotein E (*APOE*) is a major genetic risk factor for the late onset AD. ApoE, as the major cholesterol carrier in the brain, has been implicated in AD pathogenesis. However, how APOE and *APOE* isoforms directly regulate microglial functions remains largely unknown.

**Methods** Using primary culture of microglia from *Apoe* knockout (KO) mice, *APOE3* and *APOE4* targeted replacement (TR) mouse, we investigated the characteristics of microglial secreted apoE particles and the biological effects of apoE isoforms on microglial inflammatory response, migratory ability, cell viability and proliferation. Meanwhile, microglia-neuron co-culture system was utilized to study the effects of apoE isoforms on neurite outgrowth.

**Results** Herein, we found that microglia secret abundant lipidated apoE. Interestingly, apoE4 particles from primary microglia exhibited a higher lipidation status compared to apoE3 particles. Furthermore, apoE4 microglia exhibited a reduced migratory ability as well as enhanced inflammatory responses and neurotoxicity, indicating microglial apoE4 is involved in unfavourable functions.

**Conclusions** Our findings revealed the critical roles for apoE and apoE isoforms in regulating microglial functions. Our results also indicate that targeting apoE-mediated microglial inflammatory responses may serve as a potential therapeutic strategy for AD.


# 1. Background

Alzheimer's disease (AD) is the most common form of dementia, accounting for around 60–80% of all cases [1-3]. In addition to extracellular amyloid plaques and intracellular neurofibrillary tangles in the brain [4-6], emerging evidence demonstrates the critical roles of microglia in the pathogenesis of AD [7-12]. Microglia, as the resident brain macrophage, play an important role in active immune defence in the central nervous system (CNS) [13-16]. Brain glial cells are involved in the recruitment of immune cells to injury sites and initiating a cascade of inflammatory events [17]. If the inflammatory stimulus persists, they induce a chronic inflammatory state that leads to neuronal injury, dysfunction and loss [18, 19]. Additionally, microglial cells are important for normal neuron functions in the CNS and provide support to neurons as well as regulate synaptic activity [20, 21]. Altered microglial behaviour has been shown to induce neuronal degeneration in AD [22-25]. As such, understanding the functions of microglia in the brain may uncover the regulatory mechanisms for neuroinflammation and identify new therapeutic strategies for AD.

The apolipoprotein E gene (*APOE*) exists as three polymorphic alleles (ε2, ε3 and ε4) in human [26-28]. Among the three isoforms, *APOE4* is the strongest genetic risk factor for late-onset AD. *APOE4* confers an increase in toxic functions, a loss of neuroprotective function, or a combination of both in AD pathogenesis [4, 28-30]. In the brain, apoE is produced primarily by astrocytes and transports cholesterol to neurons via apoE receptors [31-33]. Previous studies have shown that astrocytes secrete several lipoprotein particles composed of phospholipid, cholesterol, apoJ and apoE, sizes of which are similar to plasma high-density lipoprotein (HDL) [34]. Astrocytic apoE3 can supply cholesterol to neurons more efficiently than astrocytic apoE4. Additionally, apoE3 primary astrocytes express higher ABCA1 protein than apoE4 primary astrocyte [35, 36]. However, the biological properties of apoE particles from primary microglia remain largely unknown. Furthermore, it has been reported that microglial *Apoe* is upregulated in AD mouse models [37-39] and that microglial activation is *APOE* isoform-dependent in AD [8]. Therefore, unveiling the role of *APOE* isoform in microglia cellular functions will help us further understand the underlying mechanisms of AD.

Herein, we characterized the biochemical properties of APOE particles from primary microglia, including the lipidation status and sizes of the particles and revealed that apoE plays an important role in regulating microglial inflammation and migration. Moreover, using a neuron-microglia co-culture system, we demonstrated that apoE4 microglia induced a higher neurotoxicity when compared with apoE4 microglia. Taken together, our findings elucidated the detrimental role of the *APOE4* isoform in microglial functions and its potential contribution to the development of AD.

# 2. Methods

## 2.1 | Animals

*Apoe* knockout (KO) mice, *APOE3* and *APOE4* targeted replacement (TR) mice, which express human apoE isoforms driven by the endogenous murine *Apoe* promoter, were purchased from Taconic. The mice were maintained at a constant temperature with an alternating 12 hr light/dark cycle. Food and water were available ad libitum. All animal experiments were approved by the Animal Ethics Committee of the Xiamen University and were conducted in compliance with all relevant ethical regulations for animal testing and research.

## 2.2 | Primary cultures

Brain tissues from C57BL6 mice were dissected for primary glial cell culture. Primary microglial cells were prepared as previously described [40] with modification. Briefly, mixed glial cells from newborn (postnatal 1 to 3 day old) pups were cultured in DMEM (GIBCO) supplemented with 10% FBS and 100 U/mL penicillin/streptomycin in a poly-D-lysine (25 μg/mL) (Sigma)-coated cell culture flasks (Corning, Fisher, USA). The medium was changed within the next day with fresh DMEM medium plus 10% FBS and 12.5 ng/mL GM-CSF (R&D). Microglial cells were harvested by shaking at a speed of 220 rpm for 15 min after 9-10 days culture. The harvested cells were seeded for experimentation or subjected to *Apoe* knockdown by electroporation.

Primary astrocytes were prepared as described by a previous protocol [41] with modification. Simply, mixed glial cells from newborn (postnatal 1 to 3 day old) pups were cultured in astrocyte culture media (DMEM, high glucose + 10% heat-inactivated fetal bovine serum + 1% penicillin/streptomycin). The medium was changed 2 days after plating of the mixed cortical cells and every 3 days thereafter. At day 9 or day 10, when astrocytes were confluent, mixed cells were shaken at 220 rpm for 30 min to remove the upper microglial cells. Trypsin (Sigma, T2601) was used to split attached astrocytes for further culture or use.

Primary cortical neurons were obtained from 11 to 17 day old embryos of wild-type C57BL/6 mice and cultured in neurobasal medium (GIBCO) supplemented with 0.5 mM GlutaMAX (GIBCO), 2% B27 (GIBCO), and 1% penicillin-streptomycin (Invitrogen) on cover glasses pre-coated with poly-D-lysine solution (50 μg/mL). At day 5 of the *in vitro* study (DIV5), the neurons were treated with 10 M cytosine arabinofuranoside (Sigma Aldrich) for 2 days to remove glial cells. At DIV7 the culture medium was then replaced with fresh neurobasal medium containing B27 and penicillin-streptomycin. For neuron-microglia co-cultures, microglia were re-suspended in neuronal culture medium and were seeded on top of primary neurons at DIV8 to a final ratio of 1:2 (microglia:neuron).

### 2.3 | Immunohistochemistry

Cells were fixed in 4% paraformaldehyde and then permeabilized with 0.25% Triton X-100 in PBS. After blocking with 1% BSA in PBS for 30 min, cells were incubated with primary antibody MAP2 (Cell Signaling, Cat#4542, 1:300) overnight at 4℃. After washing with PBS, cells were incubated with Alexa Fluor 488-conjugated secondary antibody (Abcam, ab150077) for 1 hr at room temperature. The nuclei were labeled with DAPI (Abcam, ab104139). Fluorescent signals were detected by confocal laser scanning fluorescent microscopy (Model LSM510 Invert, Carl Zeiss).

### 2.4 | *Apoe* knockdown by siRNA

Two different *Apoe* specific siRNAs (chemically synthesized by Dharmacon) were used to knockdown *Apoe* in microglia by electroporation using an Amaxa Nucleofector and a glial specific Nucleofector kit (LONZA), according to the manufacturer's instructions. Each electroporation reaction contained $4 \times 10^6$ cells and 300 nM siRNA. Transfected cells were seeded and used for subsequent experiments.

### 2.5 | Assessment of microglial migration

Microglial cells ($5 \times 10^4$) in culture medium were added to the upper well of each Transwell insert (Corning), each of which bears an uncoated filter with 8 μm diameter holes. After 48 hr, the medium in the Transwell insert was replaced with 0.2% FBS/DMEM, and DMEM containing 10% FBS, adenosine triphosphate (ATP; 300 nM), or lipopolysaccharide (LPS; 1 μg/mL) (Sigma Aldrich) was added to the lower well to induce migration. After 24 hr, the cell-bearing filters were fixed in 4% paraformaldehyde for 10 min, rinsed with PBS, and the microglial cells remaining on the upper side of each filter removed with a cotton swab. The filters were stained with 0.1% crystal violet (Sigma Aldrich) for 30 min and then rinsed

with PBS and water. The number of cells that had migrated to the underside was counted (8 random fields/filter) at 20x or 10x magnification using an Olympus DP71 microscope (Olympus, Tokyo, Japan).

### 2.6 | Reverse transcription and quantitative real-time PCR

Trizol (Invitrogen) and Direct-zol RNA MiniPrep kit (Zymo Research) were used for total RNA extraction according to the manufacturer's instructions. Reverse transcription was performed using iScript cDNA synthesis kit (Bio-Rad). The relative levels of expression were quantified and analyzed using Bio-Rad iCycler iQ software (Bio-Rad). Relative mRNA levels were calculated by $^{\Delta\Delta}$Ct method with β-actin as a reference. The primer sequences for mouse, and β-actin were as follows:

*Il-1β*-Forward: 5'-CAGGCAGGCAGTATCACTCATTG-3';

*Il-1β*-Reverse: 5'-GCTTTTTTGTTGTTCATCTCGGA-3';

*Il-6*-Forward: 5'-CAATGGCAATTCTGATTGTATG-3';

*Il-6*-Reverse: 5'-AGGACTCTGGCTTTGTCTTTC-3'

β-actin-Forward: 5'-AGTGTGACGTTGACATCCGTA-3';

β-actin-Reverse: 5'-GCCAGAGCAGTAATCTCCTTC-3'.

### 2.7 | BrdU incorporation assay

BrdU incorporation assay was performed using BrdU cell proliferation ELISA kit (Abcam) following the manufacturer's protocol. Briefly, microglial cells were seeded at a density of 1.0 x $10^4$ cells/well and BrdU was added to the cells for 48 hr for incorporation. Incorporated BrdU was examined through anti-BrdU antibodies after the cells were fixed, permeabilized and denatured. The cells were then incubated with horseradish peroxidase-conjugated secondary antibodies and the colored reaction product quantified using a microplate spectrophotometer (Varioan Flash; Thermo Fisher Scientific).

### 2.8 | Western blot analysis

All cells were lysed in RIPA buffer and total protein concentrations were determined via a BCA Protein Assay Kit (Thermo Scientific). Total protein (40 μg) was loaded into 10% SDS-PAGE. Western blot was performed as has been previously described [42]. Briefly, gels were first transferred onto PVDF membranes (Millipore). After which, primary antibodies were incubated overnight at 4°C, followed by secondary antibody incubation. The following antibodies were used in this study: Tubulin (Millipore, Cat # 05829, Host: Mouse), mouse apoE (Santa Cruz M-20, Cat # sc-6384, Host: Goat), ABCA1 (Novus, Cat # NB400-105, Host: Rabbit), ABCG1 (Novus, Cat # NB400-132, Host: Rabbit), human APOE (Meridian Life Science, Cat # K74180B, Host: Goat), GFAP (Millipore, Cat # MAB360, Host: Mouse), and Iba1 (Wako, Cat # 016-20001, Host: Rabbit). Immuno-reactive bands were detected and quantified using Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE).

### 2.9 | MTT Assay

An MTT assay was performed to assess cell viability via measuring mitochondrial activity following manufacturer's instructions (Roche). Briefly, cells were plated at a density of 5 x $10^3$ cells in a 96-well plate overnight. Cells were then incubated with MTT reagent for 4 hr at 37°C followed by the addition of developing solution to stop the reaction. The plate was read at 590 nm with a reference wavelength of 650 nm.

### 2.10 | Fast protein liquid chromatography (FPLC)

Conditioned media were generated by culturing cells in serum-free media for 24 hr in T75 flasks and then collected. Conditioned media were then concentrated 20 fold using a 10-kDa cut-off filter (Millipore) and centrifuged to remove cellular debris before storage at 4°C prior to fractionation. Samples were run through an AKTA FPLC system through a Heparin column (heparin affinity chromatography) or a Superose$^{TM}$ 6 column (size exclusion chromatography; GE Healthcare). Fractions were collected and stored at 4°C until further analysis.

### 2.11 | Cholesterol assay

Cholesterol levels were analyzed using the Amplex Red cholesterol assay kit (Life Sciences) according to the manufacturer's instructions. Briefly, samples were added into an opaque 96-well plate. Standards and samples were incubated with Amplex Red reagent (300 μM Amplex Red, 2 units/mL cholesterol oxidase, 2 units/mL HRP, and 0.2 units/mL cholesterol esterase) at 37°C for 30 min and fluorescence was measured using excitation in the range of 550 nm and emission detection at 590 nm.

### 2.12 | ApoE ELISA

A 96-well plate (Fisher Scientific) was coated overnight with WUE4 antibody [43]. After blocking in 1% nonfat milk in PBS, samples of the appropriate dilution were incubated with the detection antibody (K74180B), followed by incubation with streptavidin-poly-HRP antibodies (Fitzgerald). Finally, tetramethylbenzidine (TMB) (Sigma Aldrich) was added to each well and the substrate-peroxidase reaction was stopped by sulfuric acid stop solution. The absorbance was read at 450 nm using a BioTek 600 plate reader. ApoE concentration of samples was calculated against a standard curve derived from serial dilutions of recombinant human apoE3 or apoE4 protein (Fitzgerald).

### 2.13 | Analysis of apoE/lipoprotein particles sizes by Native PAGE

48 hr post shaking and seeding of primary cells, the medium was replaced with serum-free medium and the conditioned medium harvested and concentrated. ApoE particles in the concentrated medium were quantified and normalized to apoE3 in the conditioned medium. Then, equal amounts of apoE3 and apoE4 proteins were separated by Native PAGE Novex 4-20% Tris-Glysine gels (Thermo Fisher) under native conditions following the manufacturer's instructions and transferred to a PVDF membrane (Millipore) at 300 mA for 1.5 hr. Ponceau S staining solution (0.1% (w/v) Ponceau S in 5% (v/v) acetic

acid) was used in blotting to visualize the molecular mass markers. Particle sizes were estimated using the Native Mark Unstained Protein Standard (Invitrogen). After washing, the membrane was incubated with goat anti-apoE antibody (K74180B, Meridian Life Science) overnight at 4°C, followed by avidin-labeled donkey anti-goat antibody. Western blot bands were quantified by Image J software.

### 2.14 | ApoE associated cholesterol assay

Avidin-agarose beads (Pierce) were pre-coupled with biotinylated polyclonal anti-apoE antibody (K74180B, Meridian Life Science) and then incubated overnight with concentrated conditioned media at 4°C. Complexes of bead-antibody-apoE were washed with TBS buffer three times, then apoE associated cholesterol was eluted by 0.1% Triton X-100/TBS. 0.1 M glycine (pH 2.5) was used to elute immunoprecipitated apoE and then neutralized with 1 M Tris (pH 8.5).

### 2.15 | Quantification of neurite outgrowth and cytokines level in co-culture system

15-25 cells from 5-6 images were analysed and quantified following a method reported previously [44]. Neurite number (neurite initiation sites) and length were counted and measured using Image J. The levels of IL-1β and IL-6 in the co-culture system were examined using Quantikine ELISA kits according to manufacturer's instructions (R&D Systems, Minneapolis, MN). The absorbance was measured at 450 nm with a 590 nm correction and concentrations were calculated.

### 2.16 | Statistical analysis

All quantified data is represented as mean ± SEM. Statistical significance was determined with an unpaired *t* test, one-way ANOVA test, or two-way ANOVA and Tukey's post hoc test (GraphPad Prism 6). All experiments were performed in at least triplicate and $p < 0.05$ was considered significant.

## 3. Results

### 3.1 | Primary microglia express abundant lipidated apoE

To examine the role of apoE in microglia, we first compared the protein levels of apoE between primary microglia and astrocytes from C57BL/6 WT mice by Western blotting. Interestingly, we found that compared to astrocytes, microglia expressed higher levels of apoE (~ 12 fold) in cell lysate (Figure 1a,b). To evaluate the stability of apoE protein in these two cell types, the cells were treated with cycloheximide (CHX) to inhibit protein synthesis and harvested at indicated time points (0, 1, 2, 4 hr). Our results showed that microglial apoE was significantly more stable than astrocytic apoE (~ 70% *v.s.* ~ 10%) (Figure 1c,d), which may contribute to the above observation that primary microglia exhibited higher apoE levels than primary astrocytes.

Since APOE is a major lipid carrier in the brain, we compared the lipidation status of apoE derived from primary microglia to that of astrocytes. ApoE in conditioned medium from each cell type was purified by heparin affinity chromatography and the levels of apoE-associated cholesterol were examined. We found

that microglial apoE carries a similar ($p$=0.2125) amount of cholesterol compared to apoE derived from primary astrocytes (Figure 2a). Notably, size exclusion chromatography (SEC) showed that apoE can be detected earlier in microglia-derived conditioned medium, indicating that microglial apoE complexes exhibit increased size over astrocyte apoE complexes (Figure 2b). As ATP-Binding Cassette transporter A1 (ABCA1) is a key regulator of apoE lipidation [45-47], we next examined its expression in primary microglia. ABCA1 expression was abundant in primary microglia and significantly upregulated compared to astrocyte levels (~ 3 fold) (Figure 2c,d). Taken together, these results indicate that microglia express abundant levels lipidated apoE.

### 3.2 | Microglial apoE particles exhibit different lipidation status depending on isoforms

To investigate whether apoE particles secreted from microglia display different lipidation status, we examined the levels of ABCA1 in primary microglia derived from *APOE3*-TR and *APOE4*-TR mice, which express the human *APOE* isoform driven by the endogenous murine *Apoe* promoter. We found that apoE4 primary microglia exhibited higher ABCA1 levels (~1.6-fold) compared to apoE3 microglia (Figure 3a,b). Conversely, both the protein and mRNA levels of *APOE* were lower (protein, ~40% less; mRNA, 60% less) in apoE4 primary microglia compared to those in apoE3 microglia (Figure 3a,c,d). These results indicate that microglial apoE may be more lipidated. To directly assess whether the lipidation status of microglial apoE is isoform-dependent, we detected the level of apoE-associated cholesterol in particles isolated from apoE3 and apoE4 primary microglia. ApoE-containing particles in the conditioned medium were immunoprecipitated with a biotinylated apoE antibody and the quantities of cholesterol co-immunoprecipitated with apoE were quantified as previously described [48]. The particles from apoE4 primary astrocytes carried less cholesterol (~ 20% less) than that from apoE3 astrocytes (Sup Figure 1a) [35]. Intriguingly, apoE-containing particles from apoE4 primary microglia exhibited higher cholesterol levels (~ 1.7 fold) than those with apoE3 (Sup Figure 1b), a result consistent with the higher ABCA1 levels found in apoE4 primary microglia. Furthermore, we evaluated the sizes of apoE3 and apoE4-containing particles secreted from microglia by non-denaturing gel electrophoresis followed by Western blotting as described [48]. The sizes of apoE/lipoprotein particles were catergorized as large particles (> 690 kDa), medium particles (232-690 kDa), or small particles (< 232 kDa). Compared with apoE3 primary microglia, apoE4 microglia secreted more large particles (12% more, ratio to total apoE/lipoprotein) and less small particles (~ 10% less, ratio to total apoE/lipoprotein) (Sup Figure 1c,d). Conversely, APOE4 primary astrocytes secreted more small particles and less large particles than their *APOE3* counterparts (Sup Figure 1e,f). Collectively, our results indicate that in contrast to astrocytes, particles from microglia exhibit distinct biochemical properties depending on the *APOE* isoforms, which may play a critical role in determining their functions.

### 3.3 | *APOE4* microglia exhibits proinflammatory phenotype

It has been proposed that microglia release cytotoxic mediators and mediate inflammatory response in the AD brain [49]. To investigate the roles of *Apoe* in microglial functions, we utilized primary microglia from *Apoe*-knockout (KO) mice and established technique to knock down (KD) *Apoe* in microglia from WT

mice. As expected, apoE was undetectable in *Apoe*-KO primary microglia (Sup Figure 2a,b). We also successfully knocked down *Apoe* in WT microglia using two siRNAs targeting distinct regions of *Apoe* by electroporation (Sup Figure 2c,d). To assess whether apoE in microglia mediates immune responses, we first examined the levels of inflammatory cytokines induced by LPS in wide-type (WT) or *Apoe*-KO primary microglia. Our results demonstrated that the mRNA levels of *Il-1β* and *Il-6* were dramatically increased in *Apoe*-KO microglia treated with LPS, compared to WT microglia. Whereas, there was no difference between these cells in the absence of LPS (Figure 4a,b). Similar results were also observed in *Apoe*-knockdown (KD) primary microglia (Figure 4c,d). These results illustrate that defects in *Apoe* expression and/or function may exaggerate neuroinflammation, which could contribute to AD pathogenesis.

To further examine whether the microglial inflammatory response is *APOE* isoform-dependent, we compared the pro-inflammatory cytokine levels upon LPS stimulation in apoE3 and apoE4 primary microglia. We found that primary microglia from apoE4-TR mice were hypersensitive (*Il-1b* mRNA: apoE4, ~40-fold *v.s.* apoE3, ~28-fold; *Il-6* mRNA: apoE4, 17000-fold *v.s.* apoE3, 10000-fold) to LPS (Figure 4e,f), suggesting that apoE4 microglia exhibits a reduced ability to suppress inflammation compared with apoE3 microglia. Therefore, our studies indicate that microglial apoE plays an anti-inflammatory role upon immune stimulation, however, apoE4 microglia displays a pro-inflammatory phenotype.

### 3.4 | *APOE4* microglia exhibits reduced migratory ability

Microglia are highly motile cells that act as the main form of active immune defense in the CNS [50]. To investigate whether apoE is required for microglial migration, we first compared the migratory property of WT and *Apoe*-KO microglia using a two-dimensional “wound healing” assay [51]. We found that *Apoe*-KO primary microglia exhibited reduced migration ability (40~50% less migrated cells) compared with WT cells (Figure 5a). We next performed a three-dimensional cell migration assay using Transwell chambers (Figure 5b) with 10% FBS as a chemoattractant. This study revealed that the number of *Apoe*-KO microglia which migrated from the upper chamber to the lower chamber was significantly reduced (50% less) compared to WT microglia (Figure 5c), suggesting that the expression of *Apoe* is required for microglial mobility. To investigate whether *APOE* isoforms affect microglial migration, we performed a cell migration assay using primary microglia isolated from apoE3-TR and apoE4-TR mice (Figure 6a). It has been reported that LPS and ATP can efficiently induce microglial migration [52-54]. Thus, we examined how microglia with different apoE isoforms migrated upon the stimulation of these chemoattractants, in addition to the above used 10% FBS. Interestingly, apoE4 microglia exhibited reduced migratory activity (40-50% less) in response to the three inducers compared to apoE3 microglia (Figure 6b), indicating that apoE4 microglial migration is impaired. This impaired migration of apoE4 microglia may result in a reduced capacity for immune defense in the brain and contribute to the pathogenesis of AD.

### 3.5 | *APOE4* microglia secretes pro-inflammatory cytokines that lead to severe neurotoxicity

Previous studies suggest that microglia may exert distinct actions in response to stimuli which can be either harmful or beneficial to neuronal growth [55]. To investigate how microglia with different apoE

isoforms affect neural outgrowth, we established a co-culture system (Figure 7a1) of primary microglia and neurons to examine the effects of microglia secretions on neurite outgrowth. Neurite outgrowth was assessed by the numbers of neurite initiation sites and lengths of neurites (Figure 7a2). Co-culture assay revealed that the neurite outgrowth was suppressed when neurons were co-cultured with apoE3 or apoE4 primary microglia (Figure 7b-d), when compared with the no microglia on the top insert group (control). However, the neurites exhibited a much greater reduction outgrowth within the apoE4 microglial co-culture system that the apoE3 co-culture (Figure 7b-d). As pro-inflammatory cytokines secreted by activated microglia has been shown to inhibit neurite outgrowth [55, 56], we next examined the cytokine levels in the co-culture system. As expected, the levels of pro-inflammatory cytokines, *Il-1β and Il-6*, were increased in the system when neurons were co-cultured with either apoE3 (IL-1β, ~ 4.5 fold; IL6, ~ 7.5 fold) or apoE4 (*Il-1β*, ~ 11 fold; *Il6*, ~ 10 fold) primary microglia (Figure 7e,f). Furthermore, even both apoE3 and apoE4 microglia exibit proinflammatory response in condition of co-culture system, apoE4 microglia elicited stronger inflammatory responses with higher cytokines levels compared to apoE3 microglia in the co-culture system (Figure 7e,f). Our results indicate that apoE4 microglia result in greater neurotoxic effects by promoting neuroinflammation and this could exacerbate AD pathogenesis.

### 3.6 | *APOE* isoforms do not affect microglial viability and proliferation

Microglial proliferation and viability play important roles in neuronal protection and tissue repair [57, 58]. We thus used staurosporine [59] or GM-CSF [60, 61] as inducers to assess the effects of *Apoe* deficiency on microglial viability and proliferation. We found that *Apoe* deficiency had no effect on the viability or proliferation of microglia (Sup Figure 3a,b). Furthermore, the viability and proliferation in microglia from apoE3-TR mice were not significantly changed when compared to those in microglia from apoE4-TR mice (Sup Figure 3c,d). These results suggest that apoE may not be involved in microglial viability or proliferation phenotypes.

## 4. Discussion

Resident microglia are a specialized population of immune cells in the brain, acting as the first defense of the CNS [62]. Given that inflammation-related genes -- including *CD33* and *TREM2*, which are primarily expressed in microglia [63-67] -- have been found to be risk factors for AD, the significance of microglia in AD development is more prominent [24, 68, 69]. ApoE has been shown to be involved in AD pathogenesis, including modulation of Aβ clearance, tau pathogenesis, lipid transport, and synaptic functions [28, 30, 70, 71]. However, the roles of apoE in microglial functions have remained elusive.

Neuroinflammation induced by microglial activation is an early event and an important pathological feature in the pathogenesis of AD [69, 72, 73]. Our results showed that *Apoe*-KO primary microglia displayed exacerbated inflammatory responses, indicating the anti-inflammatory role of apoE. Thus, increasing apoE expression in primary microglia could facilitate a reduction of inflammatory responses during AD development. Additionally, apoE isoforms have been shown to differently regulate

neuroinflammatory responses. In the brain hippocampus, apoE4-TR mice showed increased glial activation in response to intracerebroventricular LPS stimulation compared to apoE2-TR and apoE3-TR mice [74]. Additionally, glial cells (~95% astrocytes, 5% microglia) from apoE4-TR mice have been found to secrete more robust pro-inflammatory cytokines than apoE3-TR mice [75]. In our study, we demonstrated that apoE4 primary microglia was more hypersensitive to LPS stimulation than apoE3 microglia, providing a mechanistic link betweenapoE4-mediated neuroinflammation and AD development. Our results are consistent with previous reports that different apoE isoforms differentially regulate the microglial inflammatory response [76]. Together, these findings indicate that apoE isoforms might impact inflammatory responses through diverse mechanisms. However, whether apoE4 from different cell types regulates neuroinflammation through its gain-of-functions in the pro-inflammatory response or loss-of-functions in the anti-inflammatory requires further investigation.\

The ability of microglia migrating to the injury sites in the brain in response to various stressors is critical to their physiological and pathophysiological actions [77]. Therefore, migration is a key component for microglia in the clearance of cell debris in tissue repair [52, 78]. Our study demonstrated thatapoE is required for microglial migration and in an apoE isoform-dependent manner, in which apoE4 microglia exhibit reduced migration toward distinct chemotaxis. This is consistent with previous evaluations, where the migration of apoE2 and apoE4 microglia were suppressed in response to complement C5a and ATP [79]. Several studies have suggested that matrix metalloproteinases (MMPs) play important roles in regulating microglial migration. ATP, specifically, induces microglial migration by regulating MMP2 and MMP9 [53, 80]. Furthermore, MMP2, MMP9, MMP12, and MMP14 have all been found to be increased in LPS-mediated microglial migration [78, 81]. It has been reported that the blood brain barrier recover from traumatic brain injury faster in apoE3-TR mice than those in apoE4-TR mice, a feature which may due to the differing levels of MMP-9 expression [82]. Whether differing apoE isoforms affect microglial migration through differentially mediating the activation of MMPs warrants further investigation.

Glial cells are critical regulators of synaptic connectivity both in healthy and diseased brains [83]. Connections between neurons have also been shown to be modified by microglia [84]. Additionally, microglia have shown the capacity to medicate synapse pruning [85-88]. One study showed that when neurons are co-cultured with glial cells (~95% astrocytes), apoE4 glia cells display a gain-of-toxic function in mediating neuronal growth [89]. However, how microglia with different *APOE* isoforms affect neurite outgrowth remains unclear. Here, we found that microglia co-cultured with neurons suppressed neurite outgrowth. Interestingly, apoE4 primary microglia secreted higher levels of pro-inflammatory cytokines in the co-culture system than their apoE3 counterparts. These findings indicate that apoE4 microglia may induce higher neurotoxicity through promoting the neuroinflammatory responses. Microglial cells have been reported to express one of the highest levels of *APOE* transcript in mice brain tissues, second only to astrocytes [90]. We also found that microglia expressed abundant APOE, which was lipidated and more stable than that from astrocytes. These results are consistent with previous studies in murine BV2 cells [91]. Intriguingly, microglial apoE4 particles were larger and exhibited a higher lipidated status than microglial apoE3 particles, which is the opposite of what was observed in primary astrocytes (i.e. apoE3> apoE4). Enhanced lipid synthesis has been reported in macrophages during

inflammation [92] and high-cholesterol diets have been shown to induce microglial activation in the hippocampus of rabbits [93], implying that higher lipid levels may increase inflammatory responses. It is possible that higher cholesterol levels carried by the *APOE4* microglia particles may contribute to their pro-inflammatory aspects, leading to enhanced neurotoxicity in AD pathogenesis.

In Tarja Malm [94], Li-Huei Tsai [95] and Julia TCW (preprint, bioRxiv, doi: https://doi.org/10.1101/713362)'s studies using human iPSC-derived microglia models, they observed a dysfunctional or impaired phenotype of *APOE4* iPSC-derived microglia when compared with that of *APOE3*, indicating that *APOE4* might contribute to AD risk via dysregulating microglia. However, the opposite conclusion has been reported in another study performed with an murine microglial cell line [96]. Our results on this contentious issue are consistent with those studies using human iPSC-derived microglia cells, although the microglia used in this work were isolated from mouse models.

There is an urgent and desperate need for and effective AD treatment [97, 98] and the targeting *APOE* has become one of the most attractive potential strategies [30, 99]. Understanding the characteristics of microglial apoE in CNS will facilitate the successful development of APOE-targeting strategies in AD treatment. Our results demonstrate the critical roles of apoE and different roles of apoE isoforms in microglial functions, yet these results need to be further verified in additional animal models. Further understanding of the molecular mechanisms and intracellular pathways underlying apoE-mediated microglial functions may shed light on how apoE and apoE isoforms contribute to AD pathogenesis and can be targeted for AD therapy.

### Conclusions

In the current study, we found that microglia-derived apoE crucially modulates microglia functions in an isoform-dependent manner. ApoE in microglia plays an anti-inflammatory role and regulates migratory behaviors of microglia without significant effects on microglial proliferation or viability. Furthermore, apoE4 primary microglia showed enhanced pro-inflammatory responses and reduced migration in response to stimuli compared with apoE3 microglia. Most importantly, apoE4 microglia exhibited strong inhibitory effects on neurite outgrowth. Our findings support a proposed model that apoE isoforms affect microglial functions, leading to subsequent neurotoxicity and dysfunction of microglial clearance (see details of schematic model in Figure 8). Together, our studies show that the characteristics of apoE from microglia have a critical role in regulating microglial functions, and demonstrate the unfavourable role of the *APOE4* allele in microglia function. These results highlight the potential for targeting apoE-mediated inflammatory responses as a therapeutic strategy for AD.

## Abbreviations

AD: Alzheimer's disease

CNS: Central nervous system

*APOE*: apolipoprotein E

HDL: high-density lipoprotein

TR: targeted replacement

FPLC: Fast protein liquid chromatography

TMB: tetramethylbenzidine

SEC: size exclusion chromatography

ABCA1: ATP-Binding Cassette transporter A1

IL-1β: Interleukin-1β

IL-6: Interleukin-6

KO: knockout

KD: knock down

# Declarations

### Availability of data and materials

The datasets used during the current study are available from the corresponding author on reasonable request.


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### Contributions

H. Z., N. W., and Y-w Z. designed the research; N. W., M. W., X. L., J. L., L. J., X. H., performed experiments; N. W., H. Z. and M. W. analyzed the data; N. W. and H. Z. wrote the manuscript. Y-w. Z. and X. X. revised the manuscript. All authors reviewed and approved the manuscript.

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### Ethics declarations

### Ethics approval and consent to participate

All animal experiments described in this study were approved by the Animal Ethics Committee of the Xiamen University and were conducted in compliance with all relevant ethical regulations for animal testing and research.

### Consent for publication

The authors are consent for the publication of this study.

### Competing interests

The authors declare that they have no competing interests.

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# Figures

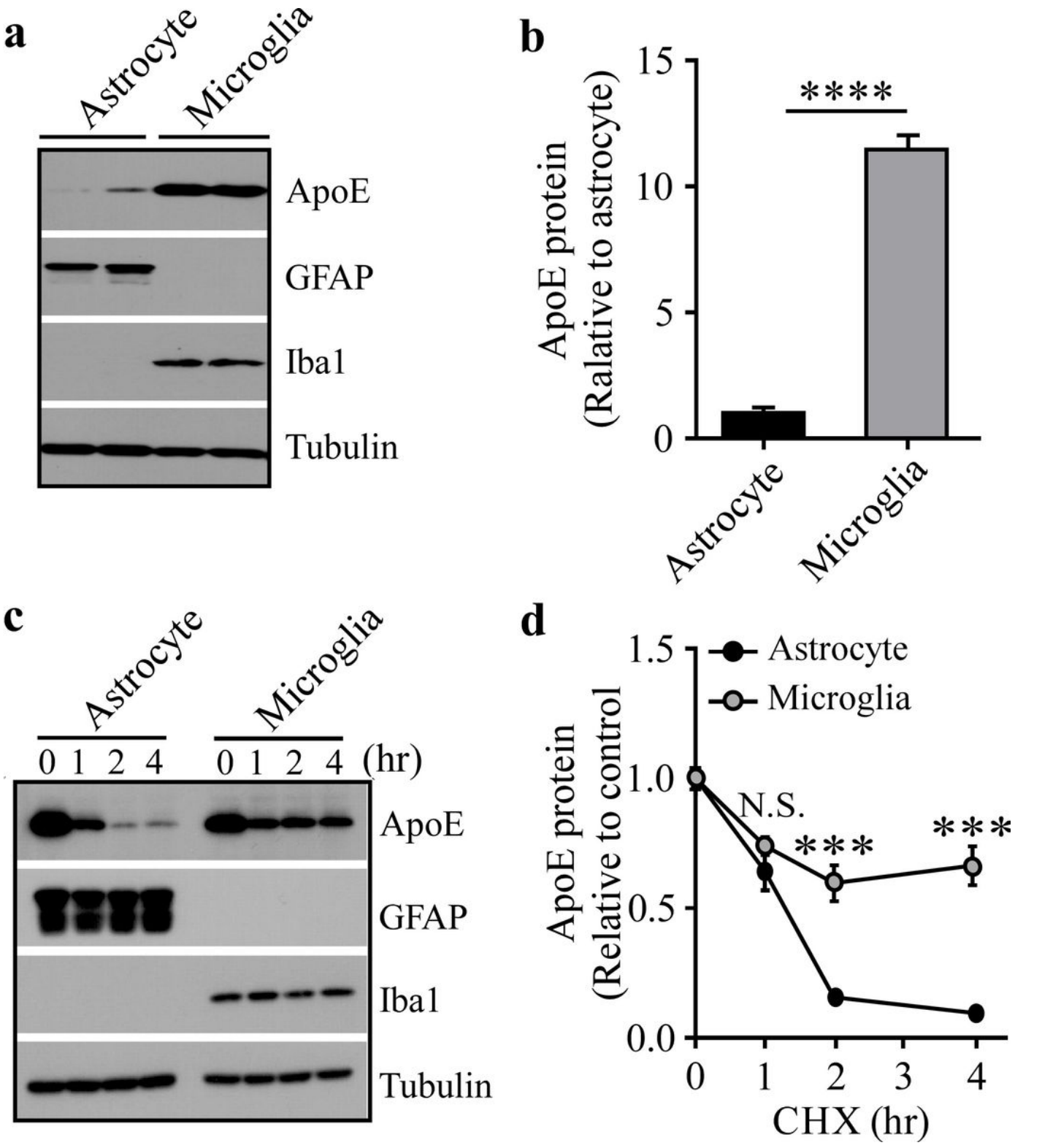
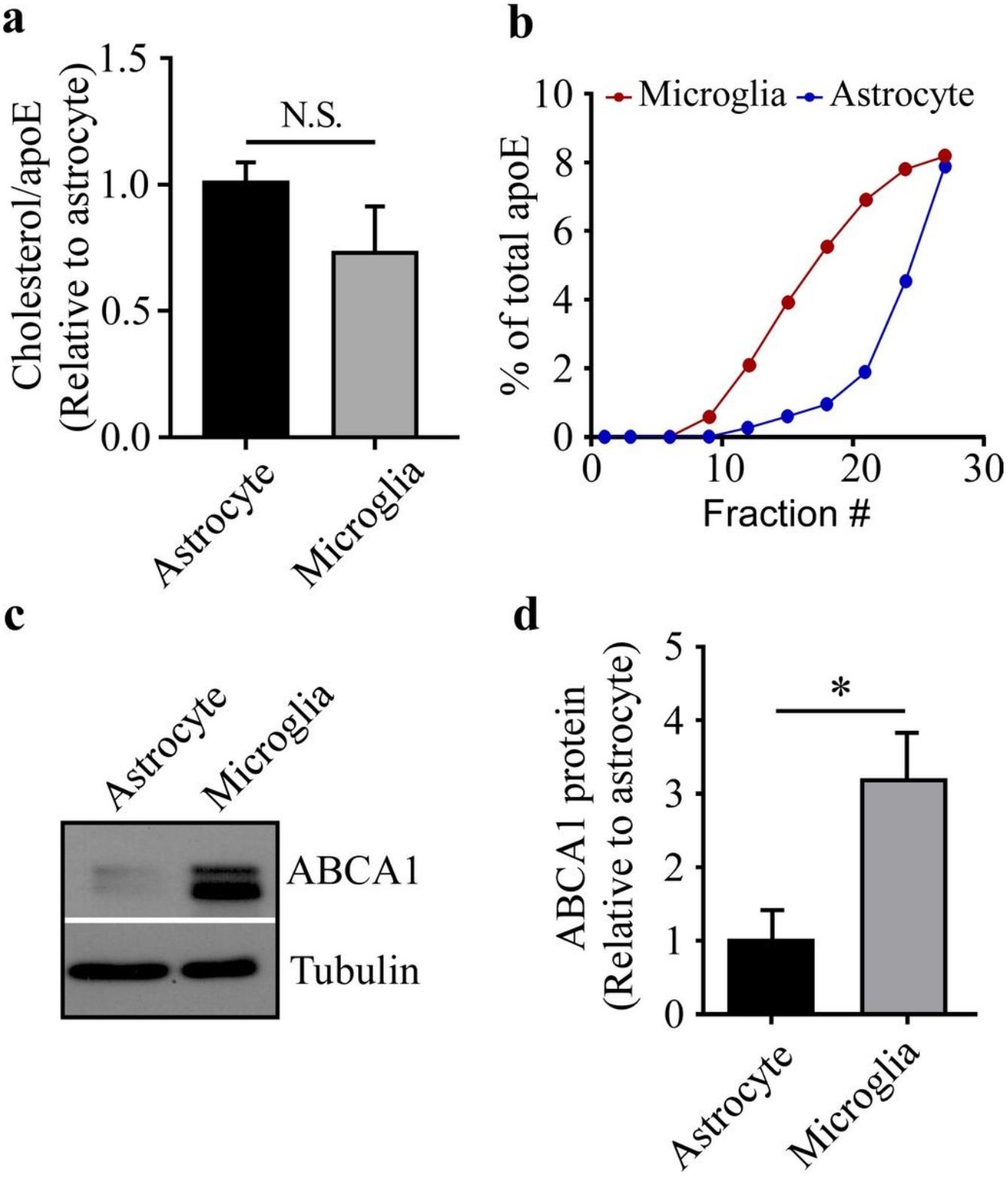



**Figure 1**

Primary microglia express abundant apoE. (a) Levels of apoE, GFAP and Iba1 in primary microglia and primary astrocyte were analyzed by Western blotting. Brain tissue from C57BL6 mice are dissected for primary glial cell culture. (b) Densitometric quantification of apoE in microglia and astrocyte. (c, d) 500 nM cycloheximide (CHX) was used to inhibit protein synthesis for the indicated period of time (0, 1, 2, and

4 hr). ApoE protein levels in microglia and astrocyte were detected by Western blotting and quantified. N.S., not significant. *, p <0.05; **, p <0.01; ***, p <0.001.




## Figure 2

Primary microglia produce lipidated apoE. (a) ApoE in conditioned medium from primary microglia and astrocytes was purified by heparin affinity chromatography. The purified apoE and associated cholesterol were examined by Western blotting and Amplex Red Cholesterol Assay. Brain tissues from C57BL6 mice

are dissected for primary glial cell culture. (b) Analysis of microglial apoE complexes and astrocyte complexes by size exclusion chromatography (SEC). (c) Levels of ABCA1 in primary microglia and astrocyte analyzed by Western blotting. (d) Densitometric quantification of ABCA1 in microglia and astrocyte is shown. N.S., not significant. *, p <0.05; **, p <0.01; ***, p <0.001.

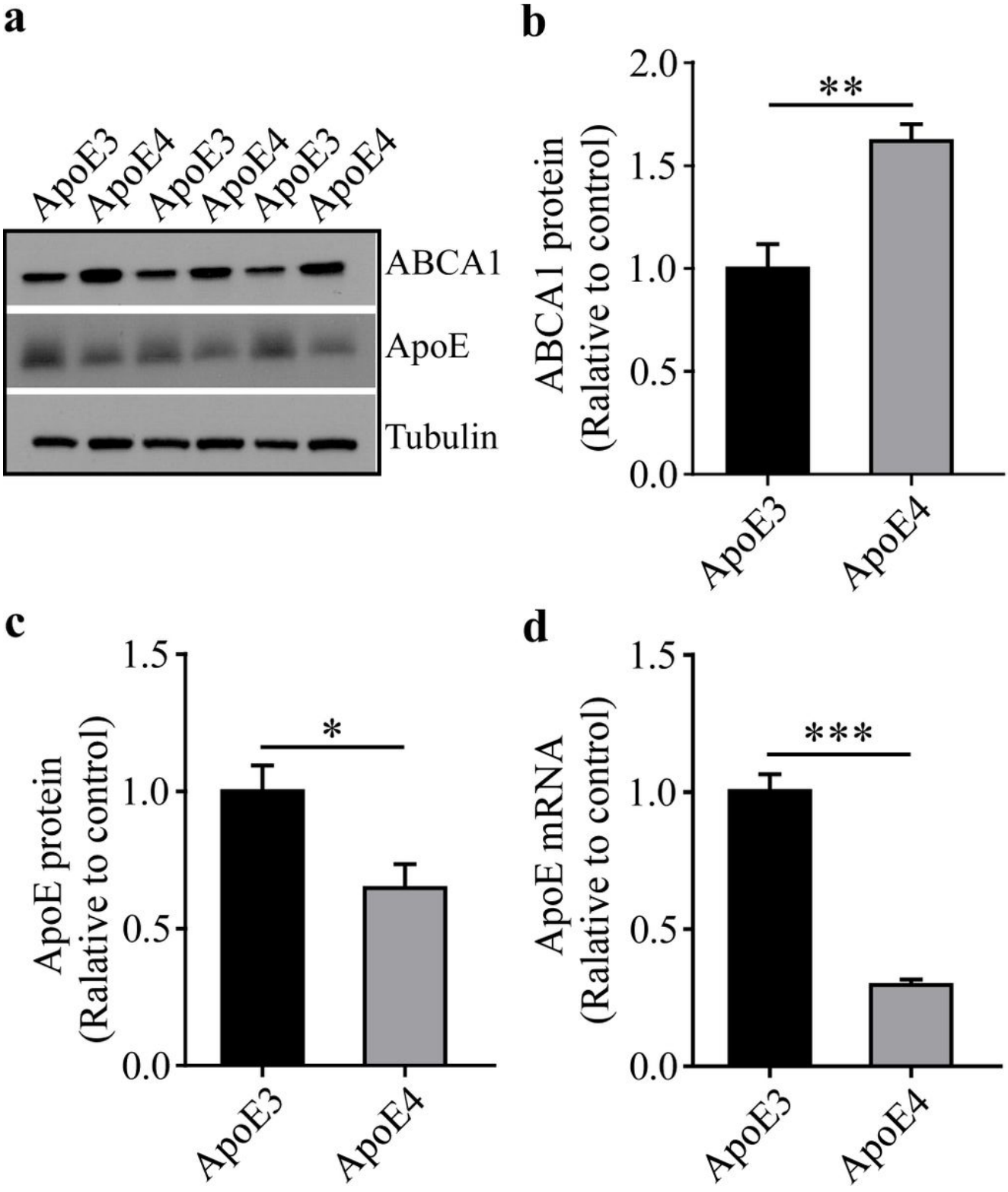



Figure 3

Increased ABCA1 and decreased apoE in ApoE4 microglia compared to apoE3 microglia. (a) Levels of ABCA1 and apoE in primary apoE3 and apoE4 microglia were analyzed by Western blotting. (b, c) Densitometric quantification of ABCA1 and apoE protein levels in primary apoE3 and apoE4 microglia is shown. (d) APOE mRNA levels in primary apoE3 and apoE4 microglia were quantified by real-time PCR. N.S., not significant. *, p <0.05; **, p <0.01; ***, p <0.001.

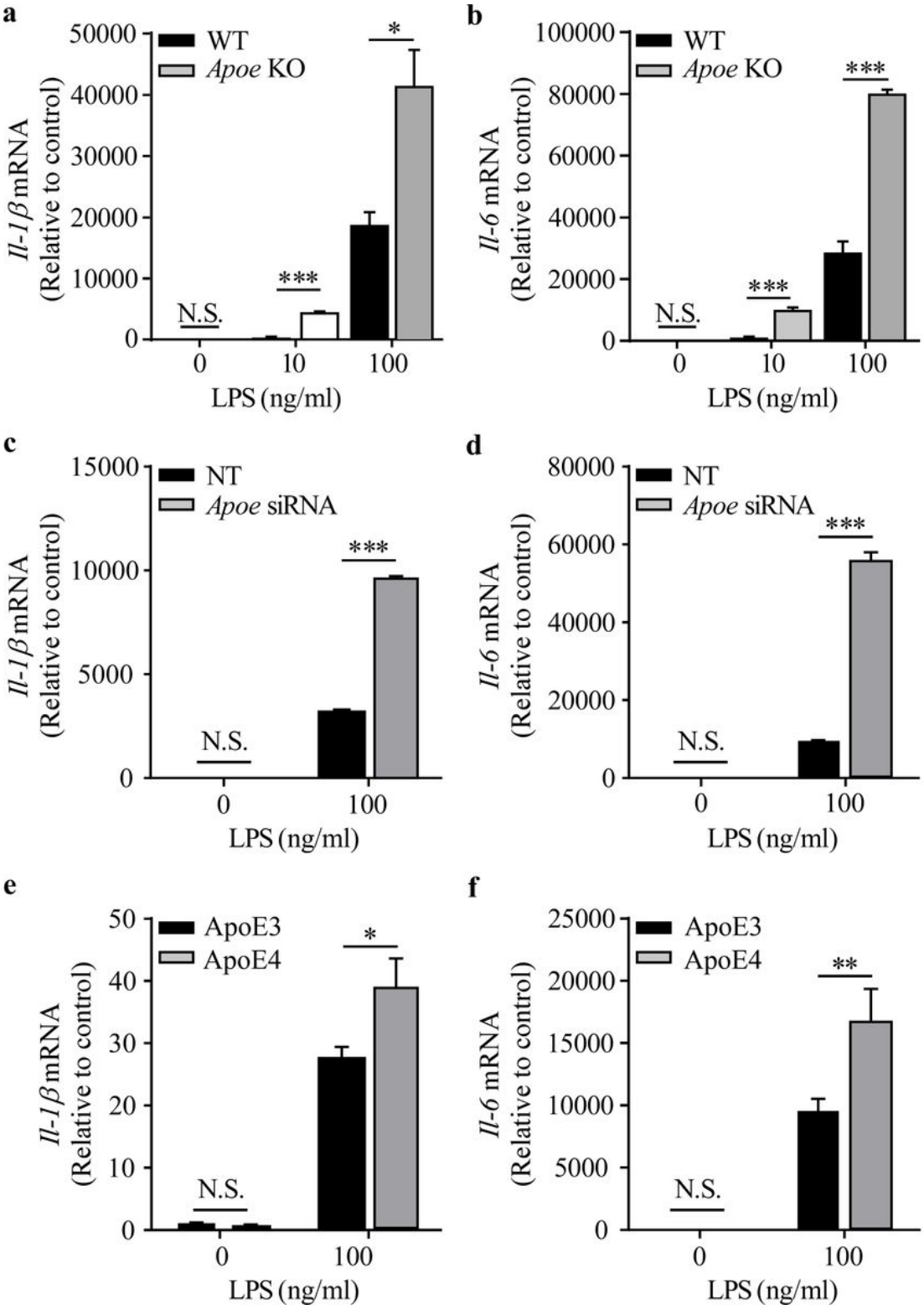



Figure 4

ApoE4 microglia exhibits enhanced proinflammatory response similar to that in Apoe deficient microglia. (a, b) Primary microglia from WT and Apoe-KO mice were treated with LPS (0–10 and 100 ng/mL) for 4 hr. All IL-1β and IL-6 mRNA levels were examined by real-time PCR. (c, d) Non-target (NT) and Apoe-KD primary microglia were treated with LPS (0 and 100 ng/mL) for 4 hr. (e, f) Primary microglia from apoE3-TR and apoE4-TR mice were treated with LPS (0 and 100 ng/mL) for 4 hr. N.S., not significant. *, p <0.05; **, p <0.01; ***, p <0.001.

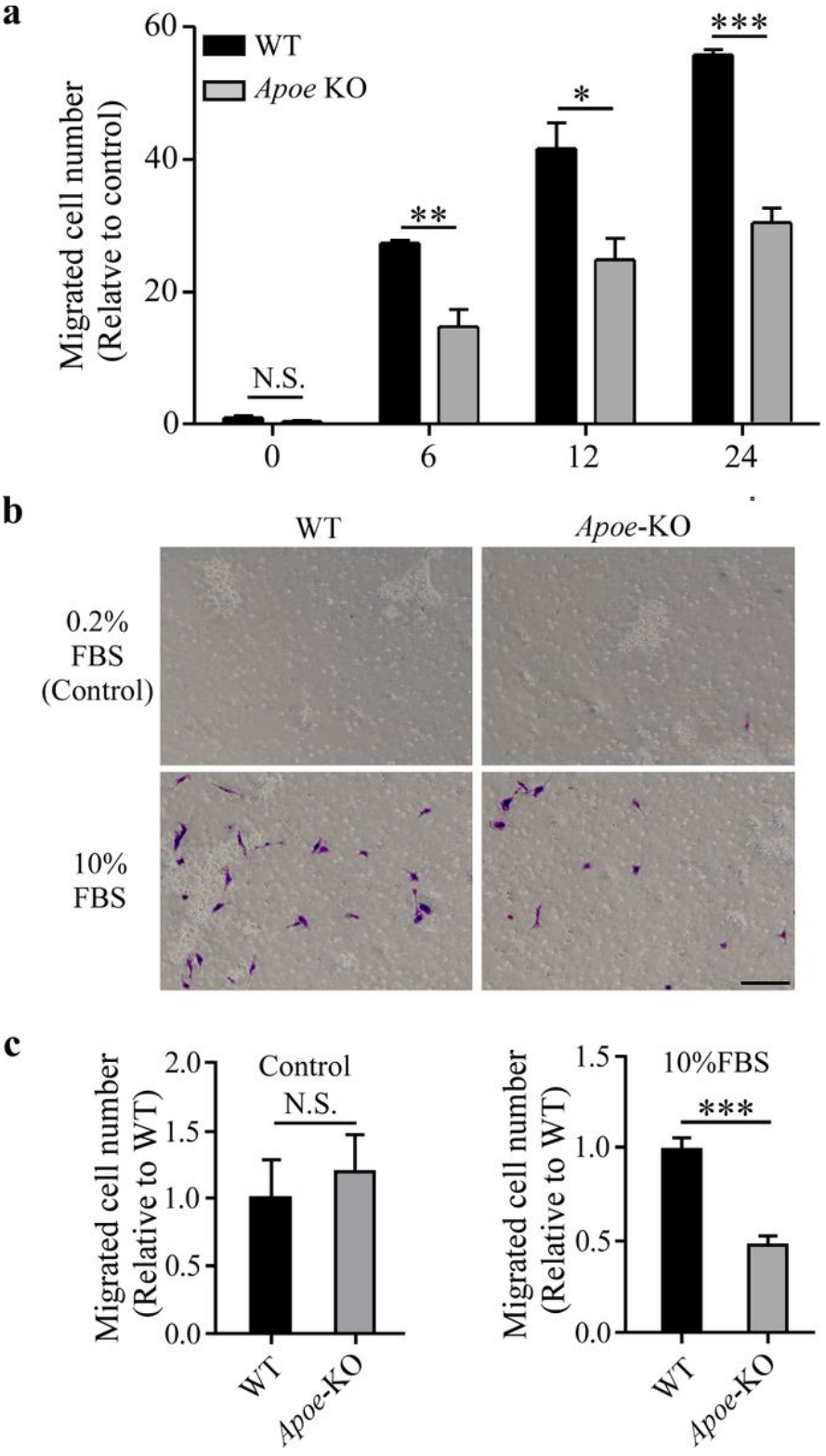
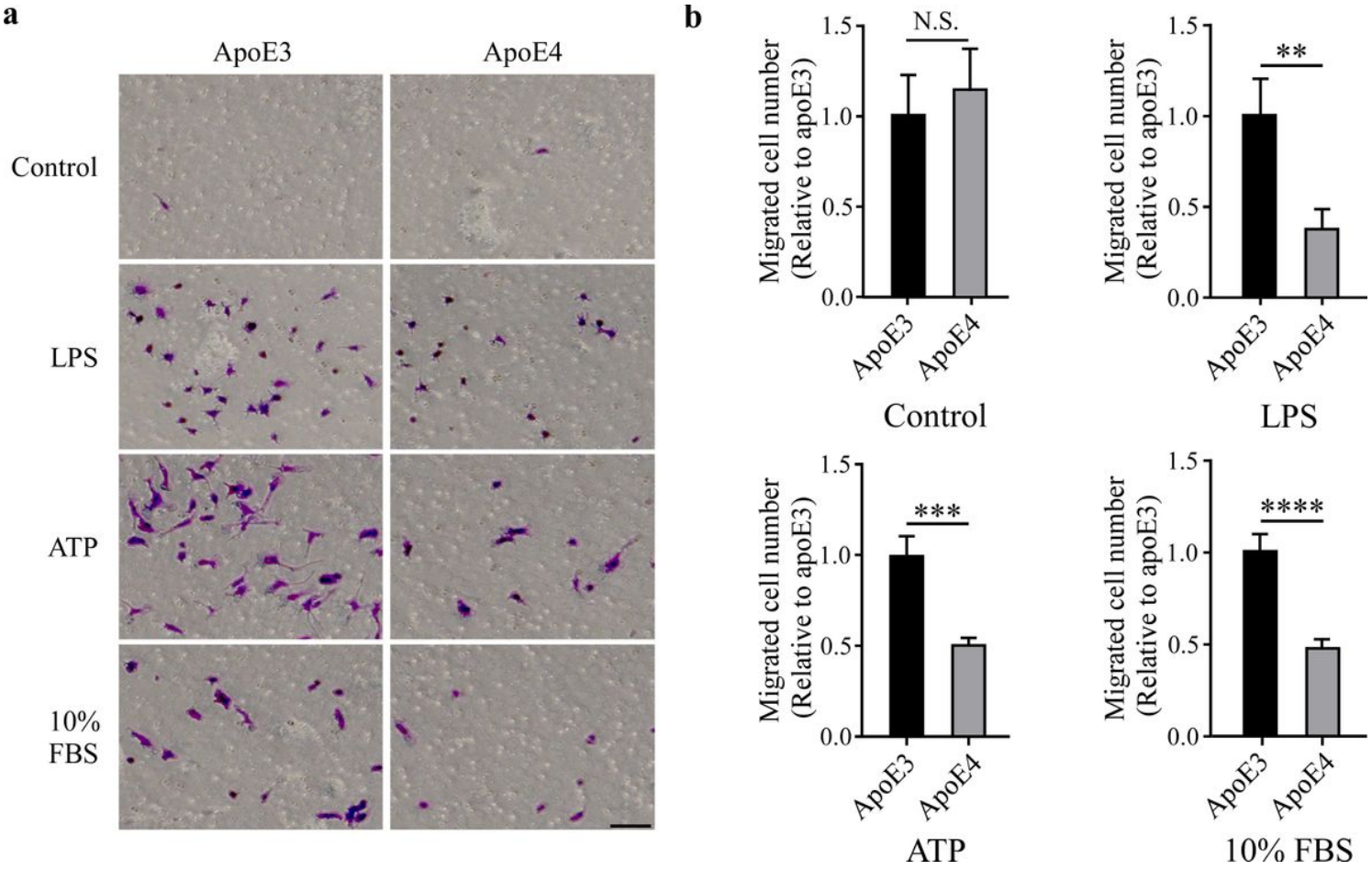


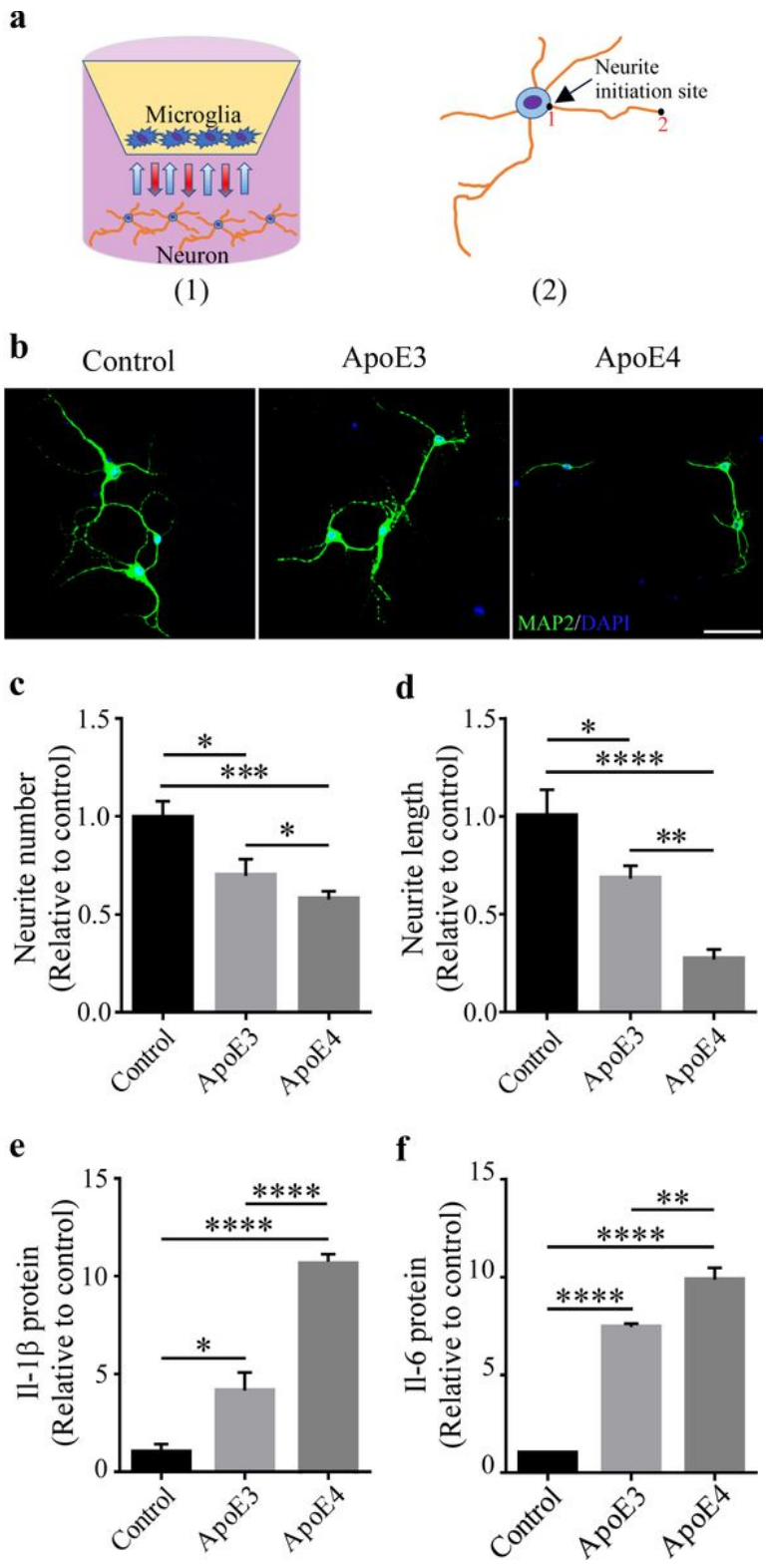

Figure 5

Apoe deficiency leads to reduced migration of primary microglia. (a) Wound healing assay was performed in WT and Apoe-KO primary microglia. Migrated cells were counted at indicated time (0, 6, 12, and 24 hr). (b) WT and Apoe-KO microglia were plated onto Transwell chamber inserts. Following 2 hr incubation with 0.2% FBS/DMEM, the medium in the lower chamber was changed to 10% FBS/DMEM or 0.2% FBS/DMEM (control) to induce microglia migration. After 24 hr induction, cells that migrated through the insert were stained with crystal violet (0.1%). Scale bar, 100 □m. (c) The number of migrated cells under each condition were counted and quantified from multiple experiments (n=8). N.S., not significant. *, p <0.05; **, p <0.01; ***, p <0.001.




## Figure 6

ApoE4 microglia exhibits reduced migration ability. (a) Microglia from apoE3-TR and apoE4-TR mice were plated onto Transwell chamber inserts. Following 2 hr incubation with 0.2% FBS in DMEM, the medium in the lower chamber was changed to 0.2% FBS/DMEM (control), 10% FBS/DMEM, LPS (1 μg/mL), or ATP (300 nM) to induce microglia migration. After 16 hr induction, cells that migrated through the insert were stained with crystal violet (0.1%). Scale bar, 100□m. (b) The number of migrated cells under each condition were counted and averaged from multiple experiments (n=8). N.S., not significant. *, p <0.05; **, p <0.01; ***, p <0.001; ****, p <0.0001.




## Figure 7

Microglia co-cultured with neurons suppress neurite outgrowth in an isoform-dependent manner. (a) Schematic diagram of microglia-neuron co-culture system (1) and neurites (2). Neurite initiation sites are counted as neurite number and is indicated by the black arrow. Neurite length is measured from the start point (● #1) to the end point (● #2). (b) Neurons co-cultured with apoE3 or apoE4 microglia were stained with MAP2. Neuronal culture with no microglia on the top insert was included as the control group. Scale

bar, 20 m. (c, d) Numbers of neurites (initiation sites) and neurite lengths were quantified and normalized to the control. (e, f) The levels of cytokines (IL-1β and IL-6) in the co-culture system were examined and normalized to the control group. N.S., not significant. *, p <0.05; **, p <0.01; ***, p <0.001; ****, p <0.0001.

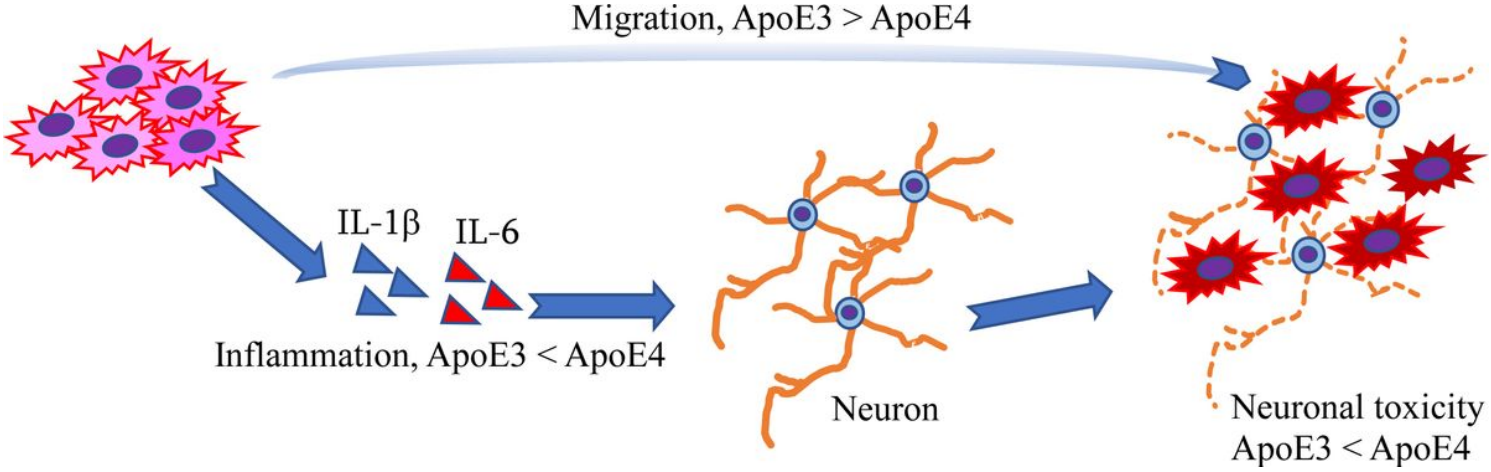



**Figure 8**

Proposed schematic diagram/model of how apoE isoforms affect microglial functions and microglia-neuron interactions. Compared to ApoE3 microglia, higher level of cytokines generated from apoE4 microglia may cause severe neurotoxicity, inducing abundant dystrophic neurites. Impaired migration of apoE4 microglia might result in lower efficiency of microglia's ability to clear dystrophic neuronal cells.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementarymaterialsWesternblottingRAWimages.docx
- SupplementaryFiguresandfigurelegends.docx