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APOE genotype-specific differences in the innate immune response

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Abstract

Apolipoprotein-E protein is an endogenous immunomodulatory agent that affects both the innate and the adaptive immune responses. Since individuals with the *APOE4* gene demonstrate worsened pathology and poorer outcomes in many neurological disorders, we examined isoform specific differences in the response of microglia, the primary cellular component of the brain's innate immune response, in detail. Our data demonstrate that microglia derived from *APOE4/4* targeted replacement mice demonstrate a pro-inflammatory phenotype that includes altered cell morphology, increased NO production associated with increased *NOS2* mRNA levels, and higher pro-inflammatory cytokine production (TNF α , IL-6, IL12p40) compared to microglia derived from *APOE3/3* targeted replacement mice. The effect is gene dose dependent and increases with the number of *APOE4* gene alleles. The *APOE* genotype-specific immune profile observed in the microglial immune response is also observed in the cortex of aged *APOE3/3* and *APOE4/4* mice treated with lipopolysaccharide (LPS) and in peripheral (peritoneal) macrophages. To determine if *APOE4*'s action resulted from an isoform specific difference in effective levels of the apolipoproteins, we generated mice expressing only a single allele of *APOE3*. Immune-stimulated macrophages from *APOE3/0* mice demonstrated an increased inflammatory response compared to *APOE3/3* mice, but less than in *APOE4/4* mice. These data suggest that inhibition of inflammation depends upon the dose of apoE3 protein available and that apoE4 protein may alter inflammation partly by dose effects and partly by being qualitatively different than apoE3. Overall, these data emphasize the important role of apolipoprotein E and of the *APOE* genotype on the immune responses that are evident in most, in not all, neurological disease.

Keywords

microglia; apolipoprotein E; innate immunity; cytokine; neuroinflammation

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1. Introduction

In the early 1980s, an elegant series of studies demonstrated that plasma lipoproteins such as apolipoprotein E (apoE) and apolipoprotein B (apoB), suppressed phytohemagglutinin-stimulated proliferation, inhibited DNA synthesis and reduced phospholipid turnover in T cells [2;20;33]. These data indicated that lymphocyte function and hence, adaptive (humoral) immunity were regulated by a subclass of apolipoproteins including apoE. Recently, apoE has also been shown to play a role in the innate immune response [3;5;8;32;38;45]. Mice that lack an *APOE* gene and apoE protein demonstrate a large increase in pro-inflammatory cytokines [14;25] suggesting that the presence of apoE regulates macrophage function.

The most prevalent variant of the *APOE* gene in the human population encodes the apoE3 protein, while other common variants that encode the apoE2 and apoE4 proteins are associated with disease [36;42]. A dose-dependent inheritance of the *APOE4* allele is associated with increased risk for developing Alzheimer's disease (AD) [12;42] and poor outcome from neurological injury [7;12;18;22;24]. Our previous studies have strongly suggested that *APOE4*'s role in AD and neurological injury may be linked, in part, to an aberrant immunomodulatory role. We have shown that macrophage function is regulated by apoE in an isoform specific manner such that the presence of the *APOE4* allele is associated with an enhanced inflammatory response compared to macrophages not expressing an *APOE4* allele [5;8;10;13]. Lynch *et al* [32] and more recently, Ophir *et al* [38] have shown that either intraperitoneal or intraventricular injection of LPS into the brains of *APOE3/3* and *APOE4/4* mice result in significantly higher levels of pro-inflammatory cytokines, defense response genes or chemotaxis genes in *APOE4/4* mice compared to *APOE3/3* mice. The higher production of cytoactive agents by *APOE4/4* microglia has been linked to an increased bystander injury to cultured neurons in the presence of activated microglia [35].

Since microglia are a primary cellular component of the brain's innate immune response, we have examined the *APOE* genotype-specific differences in microglial production of cytoactive factors in more detail. Our data demonstrate that microglia derived from *APOE4/4* targeted replacement mice demonstrate a pro-inflammatory phenotype that includes altered cell morphology and increased production of cytoactive factors. The genotype specific effects are global in nature and extend to peripheral macrophage function and to whole brain. In addition, the immune effect observed in *APOE4* mice is produced by the presence of the *APOE4* gene alleles in addition to effective changes in apolipoprotein E levels.

Methods

Reagents

1400W (Alexis Biochemicals, San Diego, CA), an iNOS specific inhibitor, was prepared in sterile 1X PBS and diluted in microglial media to a 5 μ M or 25 μ M concentration for experiments. Arginine free medium was purchased from ICN Biomedicals, Costa Mesa, CA.

Animals

Homozygous apolipoprotein E targeted replacement (*APOE* TR) mice contain a targeted insertion of exons 2–4 of the human *APOE2*, *APOE3* or *APOE4* gene that replaces the corresponding genomic DNA at the mouse *APOE* locus [44]. *APOE2/4*-TR and *APOE3/4*-TR heterozygotes were bred by crossing *APOE2/2* [44] or *APOE3/3* TR homozygotes with *APOE4/4* TR homozygotes. *APOE3/0* mice were produced by crossing *APOE3/3* to *APOE* knockout (*APOE0/0*) mice. All animals were housed under a standard light:dark cycle and received food and water *ad libitum*. All experiments were conducted in compliance with rules and regulations of the Duke University Institutional Animal Care and Use Committee.

Preparation of Microglial Cultures

Enriched microglial cultures were prepared from postnatal day 0–2 pups (gender identity unknown). Briefly, whole brains were removed and placed into sterile phosphate buffered saline (PBS) containing 100µg/ml penicillin/streptomycin and 0.5% fungizone (Invitrogen, Rockville, MD). Meninges were removed under a dissecting microscope and cortices were dissociated using the Papain Dissociation System (Worthington Biochemical Corp) as described by the manufacturer. A mixed glial culture was prepared by plating dissociated cells into 75 mm flasks and growing them at 37°C, 5% CO₂ in growth media (DMEM containing 10% bovine fetal calf serum (FBS), 2 mM glutamine and 100µg/ml penicillin/streptomycin). After 4–5 days, cultures were enriched for microglia (>95%) by replacing the growth media with fresh growth media containing 10% horse serum in place of fetal bovine serum (FBS). After an additional 3–5 days, loosely adherent cells were removed from the culture by shaking on a rotary shaker (240 rpm) for 2 hours. The cells were pelleted by centrifugation (1000g; 10 min) and resuspended into serum free medium. These cells were then plated into 96 well dishes for NO and cytokine determination or into 24 well dishes for mRNA or protein determinations. In some cases, the mixed glial culture was grown directly into 96 well dishes and the cultures enriched to microglia in the same manner. To determine the number of contaminating cells, separate control wells were periodically assessed for microglia using binding of the lectin, *Griffonia simplicifolia* (GS-1), coupled to peroxidase for cytochemical microglial identification. Color was developed using nickel-enhanced diaminobenzidine (DAB) according to manufacturer's instructions (SigmaFast DAB, Sigma) and cells were visualized using a Nikon inverted microscope (Nikon, Melville, NY). Cell viability was assessed using the MTT assay as previously described [5].

Peritoneal macrophage isolation and culture

To promote the entry of circulating monocytic cells into the peritoneum, macrophages from adult mice were elicited prior to isolation by intraperitoneal (*ip*) injection of each mouse with 5mM sodium periodate. Only adult male mice were used in these studies. After 72 hours, the mice were killed and the peritoneal cavities were flushed 2 times with warm PBS to obtain macrophages. Lavage fluid containing cells was carefully extracted from the peritoneum and the fluid containing cells from 2 to 3 mice of the same genotype was pooled. In this manner a sufficient number of macrophages were obtained for the experimental assays. The cells were pelleted by centrifugation at 1000g and then re-suspended into phenol red-free, serum-free media (high glucose DMEM containing 2 mM glutamine and 50 µg/ml gentamycin). Cells were counted, plated directly into 96 well plates and cultured for 2 days in a humidified 5% CO₂, 95% air atmosphere. During this time the macrophages attach to the plastic of the culture plate and spread, resembling typical tissue macrophages.

Immune Stimulation *in vitro*

Microglial or peritoneal macrophage cultures were immune activated by treatment with varying concentrations of recombinant mouse interferon-γ (IFNγ); polyinosinic:polycytidylic acid Poly I:Poly C (PIC), a double stranded RNA viral mimetic and/or lipopolysaccharide (LPS), a bacterial endotoxin prepared from *E.coli* serotype O11:B5 (all from Sigma, St. Louis, MO) in serum free media for 20–24 hours. Cell death was routinely measured in similarly- treated sister cultures using the MTT assay and was found to be minimal.

Immune activation *in vivo*

APOE3/3 and *APOE4/4* male mice were injected intravenously (tail vein) with 40 µg/kg body weight LPS. Mice were sacrificed at 0, 3, 7 or 17 hrs after injection and the cortex rapidly removed and frozen in liquid nitrogen. Frozen cortical tissue was pulverized on dry ice and samples were used for quantitative mRNA analysis.

Measurement of Nitrite and Protein

The supernatant levels of nitrite, a stable oxidation product of nitric oxide (NO), was used as a measure of microglial NO production. Nitrite levels were measured by chemiluminescence using a Sievers 280 Nitric Oxide Analyzer (Sievers, Boulder CO) and a nitrite standard curve prepared from sodium nitrite. Total protein ($\mu\text{g}/\text{well}$) was measured using the BCA method (Pierce, Rockford, IL) based on the manufacturer's directions using BSA ($\mu\text{g}/\text{ml}$) as standard. Nitrite levels in each well were normalized to total μg protein and expressed as nmol nitrite/ μg protein. The average background level of supernatant nitrite produced by untreated cells for each experimental group was determined and subtracted from the average immune-stimulated values.

Measurement of cytokine levels

Supernatant levels of cytokines were determined using either single cytokine ELISAs (for TNF α , IL-6 and IL-18) or the Bio-plex cytokine assay using Luminex Bead technology (for IL-1 β , IL-12p40, IL-4; IL-5, IFN γ). Single cytokine assays were carried out as described per the manufacturer's instructions (BioSource International, Camarillo, CA). TMB-One (Promega, Madison, WI) was used for substrate color development and the reaction was stopped using 1M H₂SO₄. Absorbance was measured at 450 nm using a Molecular Devices microplate reader (Molecular Devices, Sunnydale, CA).. Multiplex bead assays were performed at the Duke University Medical Center Human Vaccine Institute Core facility (under the guidance of Dr. G. Sempowski). All cytokine values were normalized to μg protein.

RNA Extraction and Preparation

RNAs from cell cultures or whole brain lysates were isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to manufacturer's instructions (Qiagen). RNA was quantified using a Beckman DV530 Life Science UV/Visible Spectrophotometer (Beckman-Coulter, Fullerton, CA) and was reverse transcribed to cDNA using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA)..

Real time PCR

Real time PCR reactions were performed using 100ng cDNA per reaction with a ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems) and Taqman Assays-on-Demand Gene Expression primer/probe sets and the Taqman Universal PCR master mix (all from Applied Biosystems) for the following mouse genes: nitric oxide synthase 2 (*NOS2*), *NOS1* and *NOS3* and eukaryotic 18S, a standard housekeeping gene.. Relative mRNA quantitation was calculated using the $2^{-\Delta\Delta\text{CT}}$ method [31] by normalizing the value of the target gene for each sample to its endogenous 18S control value, and then normalizing these values to a baseline sample, designated as the calibrator. In these experiments, the average *APOE3/3* sample served as the calibrator.

Statistical Analysis

Samples were assayed and averaged from at least 3 different litter groups (where a litter group is defined as those cells grown from a single mouse litter of 3–5 mouse pups), with a minimum of triplicate wells analyzed per each experimental paradigm for each litter group. Data were analyzed using the Student's t-test or one-way or two way analysis of variance (ANOVA) with Bonferroni's *post hoc* test where appropriate using the Prism 4.0 software package (Graphpad, San Diego, CA). Significance was set at $P < 0.05$ in all cases.

Results

Activated *APOE4/4* microglia are morphologically distinct from *APOE3/3* microglia

Activated microglia display distinct morphological features largely characterized by an amoeboid morphology and retraction of ramified processes. Bright-field micrographs of *APOE3/3* (Figure 1A) and *APOE4/4* (Figure 1B) microglia depict two distinct microglial morphologies. *APOE3/3* microglia displayed a partial ramified morphology and extended distinct cellular processes while *APOE4/4* microglia displayed an amorphous, amoeboid morphology characteristic of both *in vivo* and *in vitro* classically activated microglia [39;43].

Production of inflammatory mediators is increased in *APOE4/4* microglial cultures

To study the *APOE* genotype-specific production of inflammatory mediators, we immune stimulated microglia using polycytidylic: polyinosinic acid (PIC), a toll-like receptor -3 (TLR-3) agonist, or lipopolysaccharide (LPS), a TLR-4 agonist. Stimulation was carried out in the presence or absence of interferon γ (IFN γ), a master cytokine that helps to orchestrate inflammation [1]. After 24 hrs the supernatant levels of NO or cytokines were measured and the results were compared across genotypes. Figure 2A shows that nitrite production was significantly higher in *APOE4/4* microglia than in *APOE3/3* microglia at all doses of either PIC or LPS. The effect of the *APOE4* gene was judged as statistically significant for both types of immune activation ($p < 0.0001$) using 2-way ANOVA. The *APOE* genotype specific effect was also observed in media that contained levels of arginine similar to the level found in the brain (10 μ M) [29]. Normal tissue culture media contains supra-saturating levels of arginine (480 μ M), which is the sole substrate of iNOS. Because arginine is rate limiting for iNOS activity, the level of NO produced will change if intracellular arginine levels are reduced as a result of decreasing extracellular arginine concentrations [23]. To determine if an isoform specific difference in NO production was observed at physiological levels of arginine, we pre-incubated microglia in media containing 10 μ M arginine for 2 hrs and then immune activated the cells for 24 hrs using LPS + IFN γ . As predicted supernatant nitrite levels were decreased in cells treated with 10 μ M arginine compared to cells treated with 480 μ M arginine (Fig 2A;B). However, NO production remained significantly greater in microglia from *APOE4/4* mice in the presence of 10 μ M arginine-containing media compared to similarly treated microglia from *APOE3/3* mice (Fig 2B).

To determine if the genotype-specific effect on NO production was generated by changes in the immune-activated induction of NOS genes, we used quantitative RT-PCR to compare the levels of *NOS1*, *NOS2* and *NOS3* mRNAs in *APOE3/3* and *APOE4/4* microglia. As shown in Figure 2C, treatment for 6 hrs with 10ng/ml LPS + 10 U/ml IFN γ produced a 2.5 fold higher level of *NOS2* mRNA in *APOE4/4* microglia compared to *APOE3/3* microglia. mRNAs for *NOS1* and *NOS3* were below detectable levels. Finally, to confirm the role of iNOS in the NO produced by *APOE3/3* and *APOE4/4* microglia, we also treated immune-activated microglia with 1400W, a highly specific iNOS inhibitor (Fig 2C) [16]. Simultaneous treatment of microglia with 1400W in the presence of LPS + IFN γ resulted in a dose-dependent suppression of NO production to the same level in the *APOE3/3* and *APOE4/4* cultures, suggesting that NO production was due to iNOS in both *APOE3/3* and *APOE4/4* microglia. No effect was observed with 1400W in the absence of LPS + IFN γ .

In addition to NO, immune-activated macrophages release cytokines as part of the innate immune response [17;28]. To determine if *APOE4/4* microglia also released higher levels of cytokines, microglial supernatants were assayed for both pro-inflammatory (TNF α , IL-1 β , IL-6, IL-12p40 and IL-18) and anti-inflammatory cytokine levels using ELISA (IL-4, IL-10). LPS + IFN γ -treatment of *APOE4/4* microglia significantly increased TNF α , IL-6 and IL-12p40 levels by approximately 2.4, 2.3 and 3.3 fold, respectively compared to *APOE3/3* microglia

(Figure 3 A;B;C) while IL-4 levels were significantly lower (Fig 3D; 0.5 fold). No significant difference was observed for IL-18, IL-1 β or IL-10 (data not shown).

An increased pro-inflammatory response is also observed in peripheral macrophages

The *APOE* genotype-specific effect on immune activation is not limited to the brain but extends to the peripheral innate immune system. Peritoneal macrophages were cultured from adult male *APOE3/3* and *APOE4/4* mice and stimulated with increasing doses of PIC + IFN γ or LPS + IFN γ . A dose dependent increase was observed in PIC + IFN γ -treated cells, only. However, significantly higher supernatant levels of nitrite and TNF α were observed in macrophages derived from *APOE4/4* adult male mice compared to macrophages from *APOE3/3* mice (Fig 4).

The effect of the *APOE4* gene allele is dose dependent

The dose-dependence of the *APOE4* gene allele was examined by comparing similarly treated microglia cultured from homozygous *APOE3/3* and *APOE4/4* mice and heterozygous *APOE2/4* and *APOE3/4* mice (Fig 5). Significantly higher supernatant nitrite levels were observed in *APOE4/4* microglia compared to *APOE3/3*, *APOE3/4*, or *APOE2/4* microglia. These data suggest that the presence of the *APOE4* gene is the primary factor in the increased microglial immune responsiveness.

The effect of the *APOE4* gene on reduction of apoE protein

Ramassamy *et al* [40] have recently suggested that isoform specific differences between *APOE3/3* and *APOE4/4* expressing cells are due to a decreased overall level of apoE protein produced by *APOE4* cells compared to *APOE3* cells. To confirm and extend this finding, we measured protein expression levels in brain lysates from neonatal and from adult *APOE3/3* and *APOE4/4* mice using Western blot. We also compared untreated to LPS-treated mice to determine if immune activation affects the isoform specific differences in protein expression. As shown in Figure 6A, protein expression levels in *APOE3/3* brain lysates demonstrated significantly higher protein levels than *APOE4/4* brain lysates in both neonatal and old (60 wk) mice. Immune stimulation by LPS injection into the tail vein also did not change the relative apoE protein expression pattern. To test the effect of a reduction in apoE protein expression on the immune response of *APOE3/3* and *APOE4/4* macrophages, we generated an *APOE3/0* mouse by crossing *APOE null* (*APOE0/0*) mice to *APOE3/3* mice, thus generating a hypomorph expressing only a single *APOE3* gene allele. Brain lysates were then used to examine the expression level of apoE3 protein. Figure 6B shows representative Western blots for apoE3 protein in the brain lysates from LPS-injected *APOE3/0*, *APOE3/3* and *APOE4/4* targeted replacement mice. Protein expression levels were consistently less in the *APOE3/0* mice brain lysates than *APOE3/3* brains but *APOE3/0* levels were not significantly different from the protein expression levels in the *APOE4/4* brain. We next examined cytokine production by immune activated peritoneal macrophages derived from *APOE3/0* adult male mice and compared these results to identically treated macrophages from *APOE3/3* and *APOE4/4* adult male mice. The supernatant levels of TNF α and IL6 were significantly higher in the *APOE3/0* mice compared to *APOE3/3* mice but were significantly lower than the levels observed in *APOE4/4* mice (Fig 6C; D).

Aged *APOE4/4* mice display an enhanced CNS acute innate immune response

To test whether the *APOE4/4* genotype is associated with an enhanced neuroinflammatory response in whole animals, we measured *NOS2*, *TNF α* and *IL-12p40* mRNA expression in brains of aged (60 week old) mice subjected to LPS challenge. Following tail vein injection of LPS, we collected cortical tissue from all mice at 4 specific time points (0,3,7 and 17 hrs post injection) and determined the mRNA profiles for *NOS2*, *TNF α* and *IL-12p40* mRNA at each

time using quantitative RT-PCR. Our results showed that cortical mRNA levels for each of the pro-inflammatory genes increased significantly within 7 hours of LPS injection and returned to baseline values by 24 hrs (Figure 7A;B;C). Interestingly, *NOS2* mRNA levels did not demonstrate an *APOE* genotype specific difference. However, mRNA levels for *TNF α* and *IL-12p40* were significantly elevated in *APOE4/4* mice compared to *APOE3/3* mice. Two way ANOVA analysis revealed a significant ($p < 0.0001$) gene effect over the time course studied.

Discussion

Although *APOE4* is a well-known, statistically relevant risk factor for Alzheimer's disease [11], the widespread correlation of the *APOE4* gene with increased pathology and worsened outcomes in many diseases strongly implies that the apoE3 protein isoform plays critical roles in basic cellular mechanisms and that the apoE4 protein isoform is impaired in its ability to orchestrate these mechanisms. Multiple sites of action have been proposed for the physiological effects of apoE3 on a variety of specific cells types, including regulation of lipid distribution, maintenance of redox balance and modulation of mitochondrial energy production [36]. The impact of apoE3 on immune cells such as lymphocytes and macrophages, however, can alter disease outcome in an additional manner by affecting the overall immune status of the organism. This is clearly shown in *APOE* knockout mice that lack apoE protein. Exposure of mice lacking apoE protein to various disease-producing organisms such as *Listeria monocytogenes* and *Klebsiella pneumoniae* results in decreased resistance to infection and increased mortality [14;41]. The addition of recombinant apoE3 holoprotein or of mimetic peptides derived from the receptor binding site of apoE, both reduced LPS- or injury mediated inflammation [26;27]. Thus, these data strongly suggest that the *APOE3* gene and apoE3 protein are critical to suppressing inflammation and promoting repair.

The presence of the *APOE4* gene, in contrast, is associated with an overactive pro-inflammatory immune phenotype [5;8;19;32;38]. Our data suggest that this is an inherent property of the cells expressing *APOE4*. For example, microglia derived from *APOE4/4* mice show an "activated" morphology. Immune activated microglia are well known to assume a rounded, amoeboid-like morphology when activated both *in vitro* and *in vivo* whereas non-activated (resting) microglia show a more branched structure [39;43]. Rounded amoeboid morphology is clearly observed in untreated *APOE4/4* microglia in culture. The microglia also show an abnormal pro-inflammatory secretory profile when compared to *APOE3/3* microglia. Cytokines that are commonly found in the classical stage of an innate immune response such as NO, *TNF α* , *IL-12p40* and *IL-6* are significantly elevated in *APOE4/4* microglia compared to *APOE3/3* microglia while anti-inflammatory cytokines such as *IL-4* are significantly depressed. Jofre-Monseny *et al* [21] have recently demonstrated decreased *IL-10* levels in RAW 264.7 cells transfected with *APOE4* constructs compared to cells transfected with *APOE3* constructs. Since cytokines like *IL-4* and *IL-10* help to initiate the repair process after injury [17], these data suggest that the shift to the macrophage-orchestrated repair program is blunted or delayed in the presence of the *APOE4* gene. Finally, the effects of *APOE4* on microglial immune function depend on gene dosage. Microglia from heterozygous *APOE3/4* mice express a greater proinflammatory immune response than *APOE3/3* microglia but less than microglia from an *APOE4/4* mouse.

As predicted by the widespread immunomodulatory role of apoE3 throughout the body, the effect of the *APOE4* gene on macrophage activation is not limited to microglia but extends to macrophages in the periphery. Levels of NO and pro-inflammatory cytokines produced by peritoneal macrophages derived from *APOE4/4* adult male mice are significantly greater than the levels produced by similarly treated male *APOE3/3* mice. Our previous reports, however, have shown that the effect of the *APOE4* gene in peripheral macrophages displays gender bias [4–6;9]. A distinct genotype difference between *APOE3/3* and *APOE4/4* mice is most readily

observed in normal adult male mice but is less easily observed in adult female mice [5]. This is due, at least in part, to the exposure of adult macrophages to estrogens in female mice. Circulating levels of estrogen interact with the immune pathways regulated by apoE in a complex fashion which is different for *APOE3/3* and *APOE4/4* macrophages [4;5].

Abnormal immune modulation mediated by the *APOE4* gene is also observed *in vivo*. Our data show that LPS-injected *APOE4/4* mice display a time dependent increase in mRNA for pro-inflammatory cytokines that is significantly greater than the mRNA changes observed in brains of *APOE3/3* mice. The dramatic increase in *TNFA* and *IL-12p40* mRNA levels in *APOE4/4* mice as shown in our study mirrors the changes observed by Lynch *et al* [32] in targeted replacement *APOE3/3* and *APOE4/4* mice and by Ophir *et al* [38] using transgenic mice that express human *APOE3* or *APOE4* on a mouse knockout background. Interestingly, we do not see an *APOE4*>*APOE3* genotype- specific difference in *NOS2* mRNA expression in brain suggesting that *NOS2* mRNA levels *in vivo* do not necessarily follow *in vitro* results. This may simply be due to a technical dilution of cells expressing *NOS2* when whole brain samples are used. However, Maezawa *et al* [34] have shown that cultured astrocytes from *APOE3/3* and *APOE4/4* mice respond in the opposite manner to LPS induction compared to microglia. In astrocytes NO and cytokine production *in vitro* is greater in *APOE3* compared to *APOE4* cells [34]. These data suggest that the levels of *NOS2* and cytokine mRNA expression as measured from whole brain samples may reflect an integrated profile from multiple cell types. However, our study and that of others [32;38] clearly show higher *TNFA* and *IL6* mRNA and protein levels in brains from *APOE4/4* mice compared to *APOE3/3* mice. *NOS2* mRNA levels remain the exception and suggests that other brain- specific factors regulate *NOS2* gene expression in the brain in *APOE4/4* mice.

The mechanism by which *APOE4* differentially modulates the innate immune response is not clear. As shown by our data from *APOE3/0* macrophages, it is unlikely that reduced apoE4 protein levels compared to apoE3 protein levels are entirely responsible for the enhanced immune action observed in *APOE4/4* mice. While our data clearly show less apoE protein in *APOE4/4* mice, we still find an increased pro-inflammatory response in *APOE4/4* mice compared to *APOE3/0* mice even when the apoE protein levels are the same. Thus, the presence of the *APOE4* gene may also regulate classical immune activation and the production of pro-inflammatory factors in a manner independent of protein concentration. The pro-inflammatory effect of *APOE4* is also triggered by multiple immune receptors and may involve multiple signaling pathways. Using genome-wide expression profiling, Ophir *et al* [38] have shown that the expression of NF- κ B regulated genes in the hippocampus was significantly greater in LPS-treated *APOE4* Tg mice compared to *APOE3* Tg mice [46]. The increased expression of pro-inflammatory genes in the presence of *APOE4* compared to *APOE3* was associated with increased NF- κ B activation. A similar increase in NF- κ B activity was observed in stably transfected RAW 264.7 cells expressing *APOE4* compared to cells expressing *APOE3* [21]. The p38 MAP kinase signaling pathway is also differentially regulated in *APOE4* microglia compared to *APOE3* microglia. As recently shown by Maezawa *et al* [35], pharmacological inhibition of p38 MAP kinase abolished *APOE4*-specific toxicity in LPS-treated hippocampal slices. The finding of altered activation of the NF- κ B or p38 MAP kinase signaling pathways in *APOE4/4* microglia is consistent with our findings of increased mRNAs for *NOS2*, *IL-12p40* and *TNFA* in culture and with similar increases in whole brain lysates.

A large number of studies now show that the *APOE4* allele plays an important role in human pathophysiology. In addition to its well-characterized role in Alzheimer's disease (reviewed by [36]), very compelling genetic data show that the *APOE4* allele is associated with poor recovery from nervous system insults such as closed head injury, intracranial hemorrhage, and cardiopulmonary bypass. Other genetic associations have been reported between *APOE4* and multiple sclerosis, HIV-associated dementia, Parkinson's disease (PD), and diabetic

neuropathy (reviewed by [37]). The common thread linking these diverse disease states is an up-regulation of innate immunity, conditions where the presence of the *APOE4* gene is likely to be additive with other gene variants that collectively promote a sustained, altered immune response [30]. As discussed by Finch and Morgan [15], the presence of a global activator of innate immune function such as *APOE4*, has survival value in populations exposed to high levels of infectious disease. However, an enhanced pro-inflammatory status is clearly detrimental when faced with diseases of advanced age such as AD or PD. Thus, identification of the mechanisms underlying the increased inflammatory response in *APOE4* individuals will provide numerous insights and potential therapeutic outcomes for treating multiple inflammatory diseases.

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Figure 1. Differential morphology in *APOE3/3* and *APOE4/4* primary microglial cultures. Typical resting (non-immune stimulated) *APOE3/3* (left panel) or *APOE4/4* (right panel) microglial cultures were viewed under brightfield microscopy. Microglia from *APOE4/4* mice were consistently observed to be less branched and demonstrated a rounded, more amoeboid appearance compared to *APOE3/3* microglia.

Figure 2.

The presence of the *APOE4* gene promotes induction of *NOS2* mRNA and increased production of NO in immune stimulated microglia: **Panel A**- Average supernatant levels of nitrite ($\pm$ SEM) were measured for *APOE3/3* or *APOE4/4* microglial cultures stimulated for 24 hrs with recombinant murine IFN γ (100 U/ml) plus increasing doses of PIC or LPS. * $P < 0.04$ for IFN γ -treated *APOE4/4* microglia compared to IFN γ -treated *APOE3/3* microglia; ** $p < 0.0001$ for IFN γ + PIC- treated *APOE4/4* microglia compared to *APOE3/3* microglia; *** $p < 0.0001$ for IFN γ + LPS - treated *APOE4/4* microglia compared to *APOE3/3* microglia; all using 2-way ANOVA. A significant effect of dose was observed for PIC treatment ($p < 0.001$; 2 way ANOVA) but was not observed for LPS treatment. **Panel B**- Average supernatant levels of nitrite ($\pm$ SEM) were measured for *APOE3/3* or *APOE4/4* microglial cultures stimulated for 24 hrs with recombinant murine IFN γ (100 U/ml) + LPS (100 ng/ml) in media containing physiological levels of arginine (10 μ M). * $p < 0.001$ for *APOE4/4* compared to *APOE3/3* microglia. **Panel C**- Relative changes in mRNA for *NOS2* was determined for LPS (10 ng/ml) + IFN γ (10 U/ml)-treated *APOE3/3* microglia compared to *APOE4/4* microglia. A significant increase ($p < 0.03$; unpaired student's t test) was observed after 6 hrs of treatment in *APOE4/4* compared to *APOE3/3* microglia. **Panel D**- A significant decrease (** $p < 0.001$; 2-way ANOVA) in supernatant nitrite levels was found in both *APOE3/3* and *APOE4/4* microglia treated with LPS (100 ng/ml) + IFN γ (100 U/ml) in the presence of varying doses of 1400W, an iNOS inhibitor. No effect of 1400W alone was observed. # = $p < 0.01$ for LPS + IFN γ -treated *APOE4/4* microglia compared to *APOE3/3* microglia.

Figure 3. Cytokine production in *APOE4/4* microglia compared to *APOE3/3* microglia. Supernatant levels of TNF α (A); IL-6 (B); IL-12 p40 (C) and IL-4 (D) were measured after 24 hrs of treatment with LPS (100 ng/ml) + IFN γ (100 U/ml). Significantly increased levels for *APOE4/4* microglia compared to *APOE3/3* microglia were observed for TNF α , IL-6 and IL-12 while a significant decrease was observed for IL-4 (* = p < 0.001; unpaired student's t test).

Figure 4.

Innate immune responses are also increased in cultured peritoneal macrophages derived from adult male *APOE4/4* mice. **Panel A-** Average supernatant levels of nitrite ($\pm$ SEM) were measured for *APOE3/3* or *APOE4/4* peritoneal macrophage cultures stimulated for 24 hrs with recombinant murine IFN γ (100 U/ml) plus increasing doses of PIC or LPS. Using 2-way ANOVA, a significant effect of *APOE4* genotype (* $p < 0.001$) was observed for all treatment conditions while a significant effect of dose ($p < 0.01$) was observed for PIC treatment. **Panel B-** Average supernatant levels of TNF α ($\pm$ SEM) were measured after 24 hrs in immune stimulated *APOE3/3* and *APOE4/4* peritoneal macrophage cultures using ELISA. LPS = 100

ng/ml; IFN γ = 100 U/ml. In each case, a significantly higher level of TNF α was found in *APOE4/4* cultures compared to *APOE3/3* (* = $p < 0.001$; 2 way ANOVA).

Figure 5.

The effect of *APOE4* is dependent on the allelic dose. Average supernatant levels of nitrite ($\pm$ SEM) were measured for *APOE3/3*, *APOE2/4*, *APOE3/4* or *APOE4/4* microglial cultures stimulated for 24 hrs with recombinant murine IFN γ (100 U/ml) alone or plus LPS (100 ng/ml). A significant ($p < 0.001$, 1-way ANOVA) increase in nitrite levels was found for increasing *APOE4* gene numbers under both stimulation conditions

Figure 6.

The gene effect of *APOE4* is not solely dependent on the level of apolipoprotein E protein.

Panel A- Western blot depicting the expression of human apoE protein in brain (lanes 1–4; 7–12) and liver (lanes 5,6) lysates from *APOE3/3* and *APOE4/4* targeted replacement mice.

Lower levels of protein were observed in *APOE4/4* compared to *APOE3/3* mice regardless of age (neonatal mice – lanes 1,2, 9,10; aged to >56 wks- all other lanes) or if immune stimulated with LPS (lanes 7,8). GAPDH was used as a protein loading control. **Panel B-** Western blot

confirms a decreased expression of apolipoprotein E in LPS-treated *APOE3/0* brain lysates compared to LPS-treated *APOE3/3* brain lysates. Average relative changes in the ratio of apoE protein to GAPDH levels for each genotype are also shown (n= 4 mice/group). * = $p < 0.001$

for *APOE3/3* brain lysates compared to either *APOE4/4* or *APOE3/0* lysates. **Panel C/D-** Average supernatant levels ($\pm$ SEM) of TNF α (C) or IL-6 (D) were measured after 24hrs for immune stimulated *APOE3/3* and *APOE4/4* peritoneal macrophage cultures using ELISA. LPS = 50 ng/ml; IFN γ = 10 U/ml. * $p < 0.001$ for *APOE3/3* compared to *APOE3/0* cells; # $p < 0.01$

for *APOE4/4* compared to *APOE3/3* cells; ** $p < 0.0001$ for *APOE4/4* compared to *APOE3/3* cells; *** $p < 0.001$ for *APOE4/4* compared to *APOE3/3* cells and ## $p < 0.001$ for *APOE4/4* compared to *APOE3/0* cells.

Figure 7.

In vivo changes in the brain's innate immune response in *APOE4/4* and *APOE3/3* adult mice. Adult *APOE3/3* and *APOE4/4* mice were injected with LPS and brains harvested at 0, 3, 7 or 17 hrs after injection. Average relative changes ($\pm$ SEM) in mRNAs for *NOS2* (A), for *TNF α* (B) and for *IL-12 p40* (C) were determined using quantitative RT-PCR. Significantly higher levels of mRNA were observed for *TNF α* and *IL-12p40* (# $p < 0.001$; 2 way ANOVA).