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Apolipoprotein E Isoforms Differentially Regulate Alzheimer's Disease and Amyloid-β-Induced Inflammatory Response *in vivo* and *in vitro*

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Abstract. Neuroinflammation plays a critical role in neuronal dysfunction and death of Alzheimer's disease (AD). ApoE4 is a major risk factor of AD, while ApoE2 is neuroprotective. Little is known about the roles of ApoE isoforms in the neuroinflammation seen in AD. Their roles and mechanisms in Aβ-induced/neuroinflammation were investigated in this study using *in vivo* and *in vitro* models. Rat astrocytes were treated with lipid-poor recombinant hApoE and/or $A\beta_{42}$. Mouse astrocyte lines-expressing lipidated hApoE were treated with $A\beta_{42}$ and/or vitamin D receptor (VDR) agonist, 1α,25-dihydroxyvitamin D_3. Cells and media were harvested for cytokine ELISA, RNA isolated for qRT-PCR, and nuclear protein for transcription factor (TF) arrays and EMSA. hApoE-transgenic and AD mice were mated to generate hApoE2/AD and hApoE4/AD mice. Mice were euthanized at 6 months of age. Brain tissues were collected for cytokine ELISA array, Aβ ELISA, immunoblotting, and immunohistochemistry. hApoE4/AD mice had significantly higher levels of inflammatory cytokines than hApoE2/AD mice. Lipidated hApoE4 significantly promoted inflammatory gene expression induced by $A\beta_{42}$ but not recombinant hApoE4 in astrocytes as compared to controls. Lipidated hApoE3 provided a certain degree of protection against $A\beta_{42}$-induced inflammatory response but not recombinant hApoE3 as compared to controls. Both lipidated and recombinant hApoE2 provided protection against $A\beta_{42}$-induced inflammatory response compared to controls. TF array revealed that ApoE2 strongly activated VDR in $A\beta_{42}$-treated astrocytes. Application of 1α,25-dihydroxyvitamin D_3 completely inhibited Aβ-induced inflammatory gene expression in hApoE4-expressing astrocytes. The results suggest that ApoE4 promotes, but ApoE2 inhibits, AD/Aβ-induced neuroinflammation via VDR signaling. Targeting VDR signaling or active form of VD3 may relieve AD neuroinflammation or/and neurodegeneration.

Keywords: Alzheimer's disease, amyloid-β peptides, ApoE isoform proteins, neuroinflammation, vitamin D receptor signaling

INTRODUCTION

Neuroinflammation is one of the main pathological characteristics of Alzheimer's disease (AD), which leads to synaptic dysfunction and neurodegeneration. A number of factors are involved in initiating and propagating the cascade of the inflammatory response and signaling, such as amyloid-β

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(Aβ) peptides, hyperphosphorylated tau proteins, reactive oxygen species, metal ions, and variants of the genes involved in inflammatory response. Among these, Aβ peptides can form oligomers, aggregates, and plaques, which are toxic to neuronal cells and pro-inflammatory. Astrocytes exert influence over a range of CNS activities, including microglial-mediated neuroinflammatory responses. They respond to soluble chemical signals released from tissue during injury and disease by mobilizing to lesion sties, clearing toxic molecules, and releasing chemical signals of their own [1]. Aβ peptides are associated with the activation of microglia and astrocytes, which surround amyloid plaques and mediate the release of pro-inflammatory mediators [2]. Microglial-mediated neuroinflammation in AD brain remains an area of intense investigation, the mechanisms underlying regulation of aberrant microglial responses by astrocytes are largely unstudied [1].

Apolipoprotein E (ApoE) is a well-known regulator of cholesterol homeostasis and plays major roles in the modulation of the innate immune response [1]. A number of epidemiological, molecular, and clinical studies have demonstrated that the polymorphism of the gene encoding ApoE is associated with the risk of late-onset AD. There are three ApoE alleles, ε2, ε3, and ε4, which encode ApoE2 (cys112, cys158), ApoE3 (cys112, arg158), and ApoE4 (arg112, arg158) proteins, respectively. The ε2 allele is associated with neuroprotection for AD [3, 4], while the ε4 allele is a very strong risk factor for AD. The ε3 allele is the most frequent allele in the population and is considered to be neutral. An ε4 homozygote has ∼12 times the risk of contracting AD, compared to an ε3 homozygote [5]. While ApoE4's status as an AD risk factor is very well established, the underlying mechanisms of the risk are still largely unclear. Similarly, the mechanism of the neuroprotective effect of ApoE2 on AD also remains largely unknown. ApoE isoform-related differences have been reported at a variety of stages in Aβ processing, deposition, and degradation. In both mice and AD patients, ApoE4 is associated with higher levels of Aβ peptides and more advanced amyloid plaques in the brain, relative to ApoE3, with ApoE2 showing lower levels than the other isoforms [6–8]. Jiang et al. reported that ApoE isoforms also differed in their ability to facilitate neprilysin-mediated degradation of $A\beta_{1-42}$ within *Apoe–/–* murine microglia, with ApoE4 being least effective compared to ApoE2 or ApoE3 [9].

Little is known about the roles of ApoE, and its isoforms, in AD neuroinflammation. ApoE deficiency resulted in impaired clearance of apoptotic cells, and a systemic proinflammatory condition in mice [10, 11]. Similar reduction of ApoE in humans may contribute to a range of chronic diseases, including osteoporosis, atherosclerosis, and dementia. Studies have shown that ApoE4 allele had significantly greater systemic and brain elevations of pro-inflammatory cytokines compared with their ApoE3 counterparts *in vivo* [12], and increased levels of inflammation relative to other isoforms in cultured macrophages [13–15]. Ophir et al. treated ApoE3 and ApoE4 transgenic mice with lipopolysaccharide (LPS) and observed that ApoE3 can regulate LPS-induced astrogliosis but not ApoE4 [16]. Vitek et al. [17] reported that mice expressing one human ε3 allele (ε3/0) had lower inflammatory response upon LPS challenge than ε4/ε4 mice [17]. These studies suggest that ApoE does play a role in the inflammatory response and that ApoE4 may be pro-inflammatory while apoE2 is anti-inflammatory.

A number of studies have indicated that neuroinflammation is an important mechanism leading to synaptic dysfunction and neurodegeneration. We hypothesize that the various isoforms of ApoE protein may differentially regulate AD or Aβ-induced neuroinflammation. In this study, we show that ApoE4 promotes AD neuroinflammation while ApoE2 has a protective effect against AD neuroinflammation, acting via the vitamin D receptor (VDR) signaling pathway.

METHODS

Chemical reagents

Dulbecco's modified Eagle's medium (DMEM), Advanced DMEM, TRIzol, geneticin, sodium pyruvate, 0.25% trypsin/EDTA, and antibiotic/antimycotic were purchased from Life Technologies Inc. (Burlington, ON). Fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT, USA). Dimethyl sulfoxide (DMSO), rosiglitazone and 1α, 25-Dihydroxyvitamin D_3 were purchased from Sigma (Oakville, ON). Human recombinant ApoE isoforms were purchased from Leinco (St. Louis, MO, USA). Antibodies for NFκB, pGSK, pcJun73, and VDR were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). $A\beta_{1-42}$ and a scrambled control, featuring the same

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amino acids in a randomized order, were purchased from r-Peptide (Bogart, GA, USA).

Generation of ApoE2/AD and ApoE4/AD mice

Human ApoE2 (stock #004632) and ApoE4 (stock #004631) transgenic mice were purchased from Jackson Laboratory and mated with the APPsw/PS1dE9 (AD) mice (stock #005864) (Jackson Laboratory). Importantly, the human ApoE2 and ApoE4 isoforms in the transgenic mice are under the direction of the human glial fibrillary acidic protein and do not express endogenous mouse apoE (http://jaxmice.jax.org). The double transgenic ApoE2/AD and ApoE4/AD mice were generated and confirmed by genotyping PCR as described [18]. The double transgenic mice express an endogenous mouse apoE allele and a respective human ApoE allele (either ApoE2 or ApoE4). Also, there have been reports of gender difference in the levels of Aβ in the plasma and brains of APP/PS1 mice [19]. However, we did not observe differences in the levels of Aβ between male and female mice at 6 months of age in the animals we generated and thus there was a mixture of genders in the groups. The use of animals in this study was approved by the Animal Care Committee of the National Research Council Canada in Ottawa. The mice were maintained on a 12-h light/dark cycle in a temperature- and humidity-controlled environment with free access to food and water. Both male and female mice were used in the study.

Cell culture

The neonatal rat astrocytes (NRA) generated from the cortex of 4–8 day old Sprague-Dawley rats and immortalized using SV40 large T antigen were grown in DMEM with 10% FBS and 1% antibiotic/antimycotic and used in previous studies as described [20, 21]. Immortalized mouse astrocytes expressing human ApoE isoforms were provided by Dr. D. Holtzman from Washington University (St. Louis, MO, USA). These cells were generated and immortalized as described [22], and were grown in advanced DMEM containing 10% FBS and 200 μg/mL geneticin.

Aβ, ApoE, and VDR agonist treatments

$A\beta_{1-42}$ or scrambled $A\beta_{1-42}$ treatment of the astrocyte cell lines was performed at a concentration of 5 μM. Aβ peptide and the scrambled control peptide were brought up in 0.25% acetic acid, which served as the vehicle control. Human recombinant ApoE was brought up in 20 mM sodium phosphate +0.5 μM DTT. 1α, 25-Dihydroxyvitamin D_3 was dissolved in 99% DMSO and used at a concentration of 0.1 μM.

RNA isolation and RT-qPCR

Total RNA was isolated from treated cells using TRIzol reagent (Life Technologies) according to manufacturer's instructions. RNA samples were removed of DNA contaminates using Ambion DNA-*free* kits (Life Technologies), and RNA quality and integrity were confirmed using Experion Automated Electrophoresis Station and Experion RNA StdSens analysis kit following manufacturer's instructions (Bio-Rad). RNA was reverse-transcribed into cDNA using iScript kits (BioRad, Berkeley, CA, USA) according to manufacturer's instructions. qPCR primers were generated using Primer-Blast and ordered from IDT (Coralville, IA, USA) (Table 1). The PCR efficiency was assessed by performing standard curves using pooled cDNA material and by

Table 1
qPCR primer sequences

Gene	Sequences	
GAPDH (rat)	Forward	5′-CACTGGCATGGCCTTCCGTGTT-3′
	Reverse	5′-TACTTGGCAGGTTTCTCCAGGCGC-3′
GRO (rat)	Forward	5′-GGTCGCGAGGCTTGCCTTGA-3′
	Reverse	5′-CAGACAGACGCCATCGGTGCA-3′
IL-6 (rat)	Forward	5′-TTGCCCGTGGAGCTTCCAGGAT-3′
	Reverse	5′-AGCAGGTCGTCATCATCCCACGA-3′
GAPDH (mouse)	Forward	5′-ACCCCAGCAAGGACACTGAGCAAG-3′
	Reverse	5′-GGGGTCTGGGATGGAAATTGTGAGG-3′
GRO (mouse)	Forward	5′-CGCACGTGTTGACGCTTCCC-3′
	Reverse	5′-TCCCGAGCGAGACGAGACCA-3′
IL-6 (mouse)	Forward	5′-CTGCAAGAGACTTCCATCCAGTT-3′
	Reverse	5′-AGGGAAGGCCGTGGTTGT-3′

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plotting the log of the starting quantity of the template against the Cq values to determine the equation of the linear regression line. The BioRad SsoFast EvaGreen qPCR reaction mix was used on a CFX96 Real-time PCR detection system (BioRad) with the following conditions: 98°C for 2 min, then 39 cycles of 98°C for 2 s and 55°C for 5 s (for IL-6 reactions, 60° for 5 s was used). CFX Manager software (BioRad) was used to calculate the levels of gene expression. The gene expression levels were normalized to GAPDH.

Enzyme-linked immunosorbent assay (ELISA) kits

Commercial kits for $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ were purchased from Life Technologies (Burlington, ON). The levels of total Aβ peptides were measured in the mouse brain tissues using the kits following the manufacturer's instructions as described previously [18, 23]. Each sample was measured in duplicate and compared to linear standard curves. Total protein concentrations were determined using BCA protein assay (BioRad, Hercules, CA, USA). The concentrations of $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ in brain samples were calculated and expressed as pg/mg protein. Commercial kits for IL-6 ELISA were purchased from R&D Biosystems (Minneapolis, MN, USA) and from Life Technologies (Burlington, ON) for TNF-α. For the TNF-α assay, conditioned media from treated cells was used, while for the IL-6 ELISA, whole cell protein of the treated cells was harvested using RIPA buffer (1% NP40, 0.5% Deoxycholate, 0.1% SDS, 1X PBS). For both assays, protein levels were normalized to total protein, as measured by BCA protein assay.

Cytokine ELISA array

The mouse cytokine ELISA plate array was purchased from Signosis (Santa Clara, CA). The AD/ApoE2 and AD/ApoE4 mice (6 mice/genotype) were euthanized at 6 months of age and their brains were harvested and homogenized. Three sets of pooled homogenates were created per genotypic group (pool 2 brain homogenates x3). Applied each pooled sample to one ELISA plate (therefore, 3 plates per genotypic group), and the analysis was performed as per manufacturer's instructions. The cytokine values were corrected to the blank and the average level of each cytokine was plotted for the two groups.

Isolation of nuclear extract and protein/DNA array

NRA cells were treated with a combination of Aβ or scrambled control and recombinant ApoE isoforms. Nuclear material was isolated using a kit purchased from Panomics Inc. (Santa Clara, CA, USA). Protein concentration of each sample extract was then determined by BCA protein assay. The Protein/DNA Combo array was purchased from

Table 2
TFs changing in ApoE2 + Aβ treatment relative to the ApoE3 + Aβ treatment, as determined by Protein/DNA arrays

TF	Fold Change
VDR/DR-3	68.7
RXR/DR-1	32.9
SIE	24.6
SMAD-3/4	16.9
Stat-1	12.8
ERE	2.1
NF-E1/YY1	2.1
CP1/CTF/CBTF	–2.1
PU.1	–2.1
TFE-3L	–2.3
PPAR	–2.5
E12/E47	–3.5
AFP-1	–4.9
TEF-1/AP-5	–8.6
LH2/Lim-1	–10.3
PAX-6	–20.5
PAX-5	–20.8
TIF-1	–23.1
CP-1	–23.2
TTF-1	–31.7
IL-6-RE-BP	–42.1
CREB-2	–44.7
Stat-3(1)	–50.4
AIC/CBF	–61.5
OCT	–82.001
HOX4C	–87.589
p53	–98
X2 BP	–111.08
GATA-1	–150.8
NFkB	–159.08
c-Fos BP	–159.3
Tat	–171.4
CP-1B	–175.4
COUP-TF	–195.3
Mfh-1	–199.16
PTF-1	–216.16
NF-1/L	–238.362
TFE3	–262.843
msx-1/2/3	–375.7
SIF-2	–412.47
XBP-1 X2 BP	–522.9
PUR	–713.5
MAZ	–1006.25

TFs displaying at least a 2-fold change between ApoE2 + Aβ and ApoE3 + Aβ treatments are listed.

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Table 3
TFs found to change in the ApoE2 + Aβ treatment, relative to the ApoE3 + Aβ treatment, and which have links to AD/inflammation

TF	Relative to ApoE3 + Aβ	AD	Inflammation
Vitamin D receptor (VDR)	68.7	Y [37]	Y [49]
Retinioid X receptor (RXR)	32.9	Y [50]	Y [51, 52]
Mothers against decapentaplegic homolog (Smad)-3	16.9	Y [53]	N
Estrogen receptor element (ERE)	2.15	Y [54–57]	Y [58]
Yin Yang 1 (YY1)	2.11	Y [59]	N
Peroxisome proliferator-activated receptor (PPAR)	0.402	Y [60–63]	Y [64, 65]
Interleukin-6 response-element-binding-protein (IL-6-RE-BP)	0.023	N	Y [66]
Signal transducer and activator of transcription (STAT)-3	0.019	Y [67, 68]	Y
ApoA-I gene (AIC) promoter C region	0.016	Y [69]	N
p53	0.010	Y [70, 71]	Y [72, 73]
NFκB	6.29×10^{-3}	Y [16, 74]	Y [75]
X-box binding protein 1 (XBP-1)	1.96×10^{-3}	Y [76]	N
PUR	1.4×10^{-3}	Y [77]	N
Myc-associated zinc finger protein (MAZ)	1.22×10^{-4}	Y [78, 79]	Y [80]

Panomics Inc. (Santa Clara, CA, USA). Hybridization, probe binding and detection were performed according to manufacturer's instructions. Blots were analyzed using UN-SCAN-IT gel software (Silk Scientific, Inc, Orem, UT, USA). Three steps were used to identify the TFs of interest. The initial analysis identified the TFs that showed at least a 2-fold change in intensity between ApoE2 + Aβ and ApoE3 + Aβ treatments. The second processing step identified TFs that also did not change between the ApoE2 + Aβ scrambled and ApoE3 + Aβ scrambled treatments. The last screening step identified those TFs that also changed between the Aβ relative to scrambled treatments. This yielded 7 TFs that were upregulated and 36 TFs that were downregulated with ApoE2 + Aβ treatment as compared to ApoE3 + Aβ treatment (Table 2). This list was narrowed even further by listing only those TFs that had a reported link to either AD and/or inflammation (Table 3).

Electrophoretic mobility shift assay (EMSA)

The 3' end of the oligonucleotide DNA probes (Table 4) was labelled using the DNA 3'End Biotinylation Kit (ThermoFisher Scientific, Waltham, MA, USA), following manufacturers' instructions. Nuclear extracts were then used for the binding reactions. Thermo Scientific LightShift Chemiluminescent EMSA kits were obtained by ThermoFisher Scientific Inc. (Waltham, MA, USA) and were performed following manufacturer's instructions. To generate the supershift reactions, 2 μg of antibody was added to one of the reaction mixes. To detect the bands on the cross-linked membranes, Chemiluminescent Nucleic Acid Detection Module (ThermoFisher Scientific, Waltham, MA, USA) detection kits were used. Bands were then visualized on X-ray film at a variety of exposure times.

Immunoblotting

Total protein in the brain homogenates was resolved on 12% SDS-PAGE and transferred to PVDF membrane. The blots were probed with pGSK3β (ser9), pcJun (Ser73), and β-actin antibodies (1:1000 dilution), appropriate HRP-conjugated secondary antibodies (1:5000), and the images were quantified by densitometry and presented relative to β-actin.

Statistical analysis

Statistical analysis for all experiments was done using GraphPad Prism from GraphPad Software (La Jolla, CA, USA). For comparisons between multiple treatments, One-Way ANOVA was used, with *post-hoc* analysis using the Bonferroni method.

Table 4
DNA probe sequences for EMSA reactions

Target	Sequences	
NFκB	Sense	5′-TTTCGCGGGGACTTTCCCGCGC-3′
	Anti-sense	5′-TTTGCGCGGGAAAGTCCCCGCG-3′
VDR	Sense	5′-AGCTTCAGGTCAAGGAGGTCAGAGAGC-3′
	Anti-sense	5′-GCTCTCTGACCTCCTTGACCTGAAGCT-3′

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For comparisons between single treatments, Student's t-test was used.

RESULTS

An association exists between ApoE4, AD, and neuroinflammation

Our previous study had shown that pro-inflammatory cytokines were significantly increased in AD brains relative to age-matched non-demented (ND) controls [24]. Of note, 40–70% of the AD patients carry the ApoE4 allele whereas most ND control individuals carry the ApoE2 and/or ApoE3 alleles. These findings highlight a complex relationship between ApoE4, AD, and inflammation. This APOE isoform-modulated Aβ-induced neuroinflammation is a very important factor in AD [21]. To determine the contributions of ApoE2 and ApoE4 to AD phenotypes, particularly in inflammation, we analyzed inflammatory phenotypes from ApoE2 and ApoE4 alleles both in the *in vivo* and *in vitro* models. First, the brains of ApoE2/APPsw/PS1dE9 (ApoE2/AD) and ApoE4/APPsw/PS1dE9 (ApoE4/AD) mice were assessed for inflammatory markers. ApoE2/AD and ApoE4/AD mice (males and females) were generated and sacrificed at 6 months of age. Cytokine ELISA arrays were performed on brain tissue lysates from the mice and revealed that the levels of IFNγ, MCP-1, MIP-1α, SCF, and RANTES were significantly higher in the brains of ApoE4/AD relative to ApoE2/AD mice (Fig. 1, two-tailed t-test, $^{*}p < 0.05$, $^{**}p < 0.01$). Based on the amyloid-hypothesis, that increased Aβ is observed in AD patients, it was next assessed whether Aβ levels were altered in ApoE4/AD compared to ApoE2/AD mouse brains. $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ ELISAs revealed that homogenized brains of ApoE4/AD mice exhibited increased levels of $A\beta_{1\text{-}42}$ relative to ApoE2/AD mice (Fig. 2A, left panel, two-tailed t-test, $^{*}p < 0.05$), but the levels of $A\beta_{1\text{-}40}$ were not significantly altered from ApoE4/AD versus ApoE2/AD expressing mice (Fig. 2A, middle panel) resulting in a slight but insignificant increase in the $A\beta_{42}/A\beta_{40}$ ratio for AD/ApoE4-containing mice (Fig. 2A, right panel). Aβ peptides can activate the p38MAPK [20], NFκB [21], and JNK-AP1 [24] signaling pathways and modulate GSK3β activity for tau pathology. Thus, we evaluated the levels of phosphorylated c-Jun Ser73 and phosphorylated GSK3β ser9 in the brains of ApoE4/AD relative to ApoE2/AD by western blotting. The data show that AD/ApoE4 mice displayed decreased levels of pGSK3β (Fig. 2B, two-tailed t-test, $^{**}p < 0.01$), and increased levels of p-cJun/ser73 (Fig. 2C, two-tailed t-test, $^{*}p < 0.05$), relative to AD/ApoE2 mouse brain. The results show that ApoE4/AD mice exhibit higher levels of inflammatory cytokines and the JNK-AP1 signaling pathway (that activates inflammatory response) than ApoE2/AD mouse brains. ApoE4/AD mouse brains also display decreased GSK3β phosphorylation at ser9 (indicating an increased activity of the enzyme for tau phosphorylation) and increased levels of neurotoxic $A\beta_{1\text{-}42}$ peptides. To gain an understanding of the underlying mechanisms of ApoE isoforms and Aβ-induced inflammation we used *in vitro* models.

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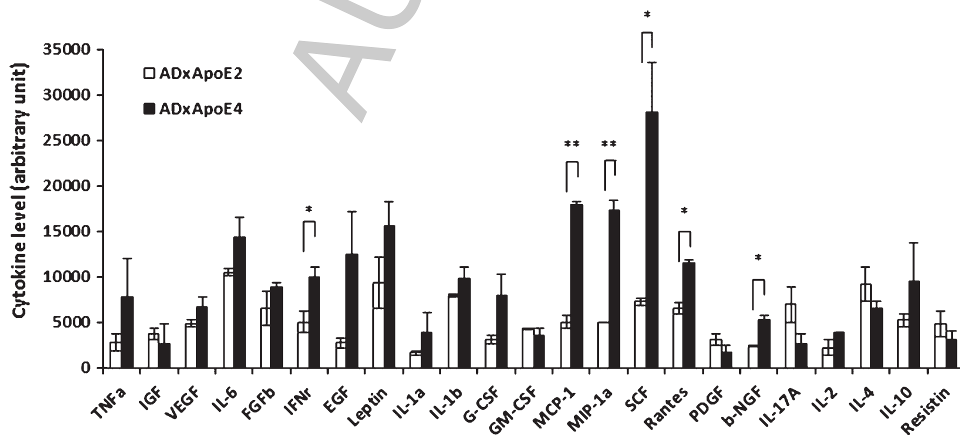

Fig. 1. Cytokine levels in ApoE2/AD and ApoE4/AD mouse brains. ApoE2/APPsw/PS1dE9 and ApoE4/APPsw/PS1dE9 mice (6 mice/group) were sacrificed at 6 months of age, and the brains were harvested and homogenized. The brain homogenates were applied to the mouse cytokine ELISA plate array, and the average level of the cytokines were plotted. Two-tailed t-test, $^{**}p < 0.01$, $^{*}p < 0.05$.

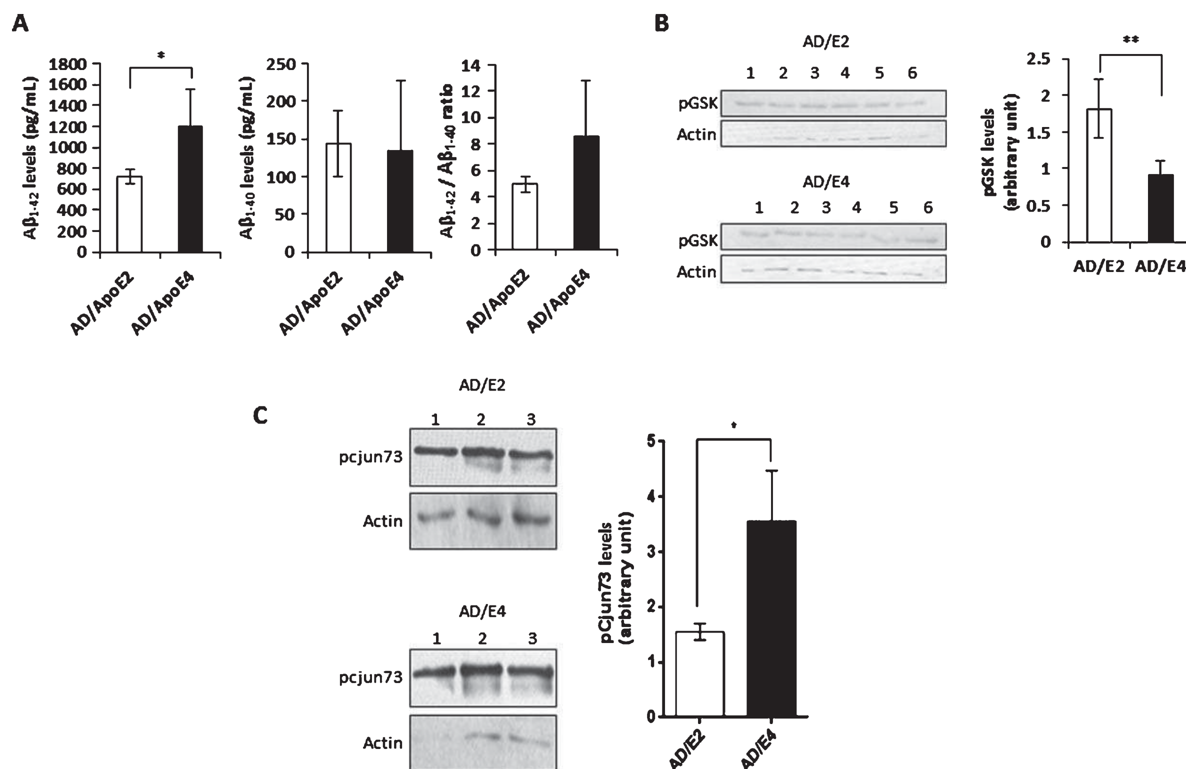

Fig. 2. Levels of $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ peptides, pGSK, and pc-Jun73 in the brains of ApoE2/AD and ApoE4/AD mice. ApoE2/APPsw/PS1dE9 and ApoE4/APPsw/PS1dE9 mice (6 mice/group) were sacrificed at 6 months of age, and the brains were harvested. A) Brain homogenates were applied to $A\beta_{1\text{-}42}$ and $A\beta_{1\text{-}40}$ ELISA kits, and the levels of $A\beta_{1\text{-}42}$ and $A\beta_{1\text{-}40}$ (pg/ml) were normalized to total brain protein, averaged and plotted (left and middle panels). The $A\beta_{42/40}$ peptide ratio was also calculated and plotted (right panel). B) Total protein in the brain homogenate was solubilized by sample loading buffer, resolved on SDS-PAGE and transferred to PVDF membrane. pGSK and actin were detected by western blotting (left panel), and quantified by densitometry and presented relative to actin (right panel). C) To save on antibody, brain homogenates 1&2, 3&4, and 5&6 were pooled from each group (AD/E2 and AD/E4) to create new homogenates 1, 2, and 3, respectively. The brain protein was solubilized by sample loading buffer, resolved on SDS-PAGE and transferred to PVDF membrane. pcJun73 and actin were detected by western blotting (left panel), and quantified by densitometry and presented relative to actin (right panel). Two-tailed *t*-test, $^{**}p < 0.01$, $^{*}p < 0.05$).

ApoE isoforms modify Aβ-induced inflammatory response in vitro

Neonatal rat astrocytes (NRA) were used to study the relationship between ApoE isoforms and $A\beta_{1\text{-}42}$-induced inflammation *in vitro*. To show that Aβ activated inflammation in NRAs, the cells were treated with $A\beta_{1\text{-}42}$ and the expression levels of pro-inflammatory cytokines were detected by RT-qPCR. NRA cells were treated with 5 μM $A\beta_{1\text{-}42}$, scrambled $A\beta_{42\text{-}1}$ peptide or vehicle for 6 h, and the levels of GRO and IL-6 were measured by RT-qPCR. Expression of GRO was significantly increased upon Aβ challenge, relative to both vehicle and scrambled Aβ controls (Fig. 3A, left panel, One-way ANOVA, Bonferoni *post-hoc* test, $^{**}p < 0.01$). Expression of IL-6 was also significantly increased in the presence of Aβ relative to scrambled Aβ (Fig. 3A, right panel, One-way ANOVA, Bonferoni *post-hoc* test, $^{*}p < 0.05$). Thus, $A\beta_{1\text{-}42}$ elicited an inflammatory response in NRA cells as evidenced by the increase in expression levels of pro-inflammatory cytokines GRO and IL-6, among others (data not shown), and as such the levels of these two cytokines were used as markers of inflammation in this study. Next, it was examined whether various isoforms of recombinant ApoE alter the Aβ-induced inflammatory response. NRA cells were treated with one of the three isoforms of recombinant human ApoE (E2, E3, or E4) (lipid-poor form of ApoE) at a concentration of 3 μM for 24 h, and then 5 μM $A\beta_{1\text{-}42}$ was added for 6 h, and inflammation was assessed by measuring the expression levels of the pro-inflammatory cytokines GRO and IL-6. There was a trend with ApoE2 + Aβ treatment exhibiting reduced expression of GRO relative to ApoE3 + Aβ, ApoE4 + Aβ or

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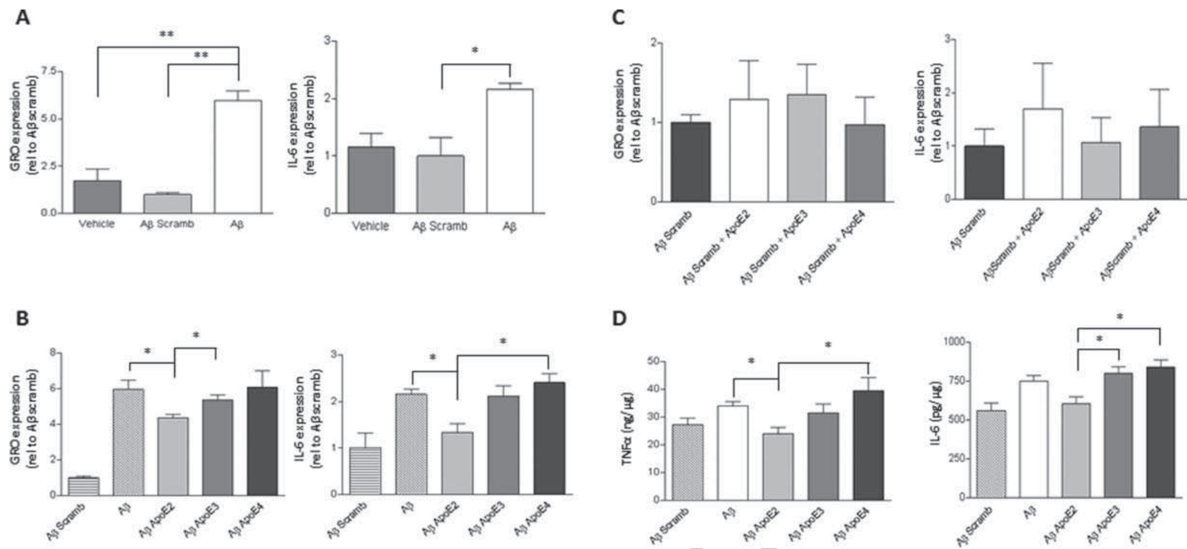

Fig. 3. The effects of $A\beta_{1-42}$ peptides and ApoE isoforms on inflammatory gene expression in NRA cells. A) NRA cells were treated with 5 μM $A\beta_{1-42}$, scrambled Aβ or vehicle for 6 h. Expression of GRO (left panel) and IL-6 (right panel) were determined by RT-qPCR, normalized to GAPDH and plotted (One-way ANOVA, Bonferroni *post-hoc*, $^{**}p < 0.01$, $^{*}p < 0.05$, $n = 3$). B) NRA cells were treated with ApoE isoforms at 3 μM for 24 h, and then with $A\beta_{1-42}$ at 5 μM for 6 h. Expression of GRO (left panel) and IL-6 (right panel) were determined by RT-qPCR, normalized to GAPDH and plotted ($n = 3$) (One-way ANOVA, Bonferroni post-hoc, $^{*}p < 0.05$). C) Cells were treated as described in B, but with scrambled Aβ instead of $A\beta_{1-42}$. Expression of GRO (left panel) and IL-6 (right panel) were determined by RT-qPCR, normalized to GAPDH and plotted. D) Cells treated as described in B. Conditioned media was applied to TNF-α ELISA and the TNF-α levels were normalized to total cellular protein, and presented as ng/μg protein ($n = 3$) (left panel). Whole cell lysate was applied IL-6 ELISA and the levels of IL-6 were normalized to total cellular protein, and presented as pg/μg protein (right panel) (One-way ANOVA, Bonferroni post-hoc, $^{*}p < 0.05$, N = 3).

to Aβ alone (Fig. 3B, left panel). There was no difference in GRO expression between ApoE3 + Aβ or ApoE4 + Aβ and Aβ treatment alone. ApoE2 + Aβ treatment also exhibited a trend of reduced expression of IL-6 relative to Aβ alone or ApoE4 + Aβ treatments (Fig. 3B, right panel). In the absence of $A\beta_{1-42}$, there was no difference in GRO or IL-6 expression between scrambled Aβ and the ApoE isoform treatments (Fig. 3C). To determine if Aβ and ApoE isoforms treatments also affected protein levels of inflammatory markers, media and cell protein were harvested from treated NRA cells, and TNF-α and IL-6 were measured, respectively, by ELISA. TNF-α levels in media collected from cells treated with ApoE2 + Aβ showed a trend to be reduced as compared to the ApoE4 + Aβ treated cells or to Aβ treatment alone (Fig. 3D, left panel). IL-6 levels in whole cell lysate exhibited a trend of reduction in the ApoE2 + Aβ treated cells relative to ApoE3 or ApoE4 + Aβ treated NRA cells (Fig. 3D, right panel). These data show that ApoE isoforms can alter the Aβ-induced inflammatory response in NRA cells. The presence of recombinant ApoE2 seems to reduce the Aβ-induced inflammatory gene expression while the presence of ApoE3 or ApoE4 does not. The changes in inflammatory gene expression in response to different ApoE isoforms were not exclusively an RNA effect, but did ultimately result in changes in protein levels of these cytokines.

Immortalized mouse astrocytes, with native murine apoE knocked-out and human ApoE isoforms knocked-in, were also used as an *in vitro* model to test whether lipidated ApoE isoforms modify Aβ-induced inflammation. The mouse astrocytes expressing hApoE isoforms were challenged with $A\beta_{1-42}$ under the same conditions used for NRA cells. Each of the three cell lines showed a trend of increase in GRO and IL-6 expression upon $A\beta_{1-42}$ treatment (Fig. 4). In ApoE4-expressing cells, the increase in GRO expression upon $A\beta_{1-42}$ treatment was significant relative to scrambled Aβ (Fig. 4A, one-way ANOVA, Bonferoni *post-hoc* test $^{*}p < 0.05$). IL-6 expression was significantly increased in ApoE4-expressing cells upon $A\beta_{1-42}$ treatment relative to the scrambled control (Fig. 4B, one-way ANOVA, Bonferoni *post-hoc* test $^{*}p < 0.05$). ApoE4-expressing cells displayed significantly higher IL-6 expression after Aβ challenge, compared to ApoE3 + Aβ or

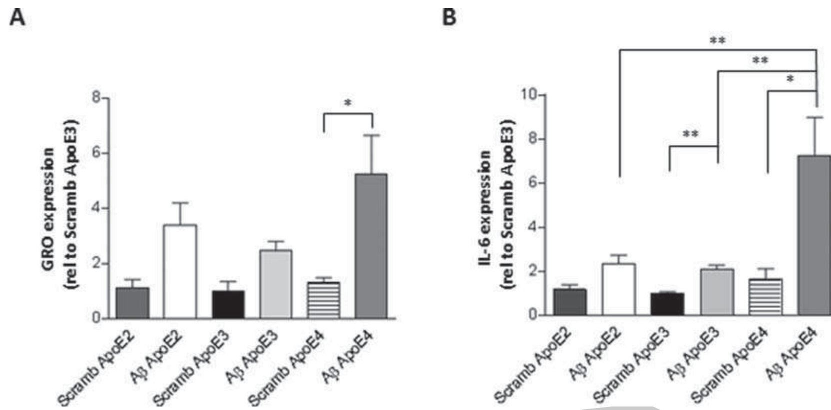

Fig. 4. Inflammatory gene expression in mouse apoE knock-out, human ApoE knock-in, astrocytes upon challenge with $A\beta_{1\text{-}42}$. Mouse astrocytes expressing either hApoE2, E3, or E4 were treated with 5 μM $A\beta_{1\text{-}42}$ for 6 h. Expression of GRO (A) and IL-6 (B) were determined by RT-qPCR, normalized to GAPDH and plotted (one-way ANOVA & Bonferroni *post-hoc*, $^*p < 0.05$, $^{**}p < 0.01$).

ApoE2 + Aβ (Fig. 4B, One-way ANOVA, Bonferroni *post-hoc* test, $^{**}p < 0.01$). A similar trend was observed with GRO expression (Fig. 4A). IL-6 expression upon $A\beta_{1\text{-}42}$ treatment in the ApoE2-expressing mouse astrocytes was not significantly increased relative to the scrambled control (Fig. 4B). Together the data from the NRA and mouse astrocytes suggest that ApoE2 can reduce Aβ-induced inflammatory response, while ApoE3 and mainly ApoE4 can exacerbate the inflammatory response.

Signaling pathways are differentially activated by ApoE isoforms and Aβ combination treatments

To determine if the differential signaling pathways are activated from Aβ + ApoE2 versus Aβ + ApoE3 treatments, nuclear materials from treated NRA cells were run on Protein/DNA Combo TF arrays. Densitometry analysis of the blots revealed the levels of activation of each TF from each treatment (Fig. 5). The TF arrays identified 7 TFs that were upregulated, and 36 TFs that were downregulated in cells treated with ApoE2 + Aβ as compared to the cells treated with ApoE3 + Aβ (Table 2) (refer to Materials and Methods section for selection criteria). Table 3 lists those TFs that were also reported to be associated with AD and/or inflammation; this includes 5 TFs (VDR/DR-3, RXR/DR-1, SMAD-3/4, ERE and NF-E1/YYI) that were upregulated and 9 TFs (PPAR, IL-6-RE-BP, STAT-3, AIC, p53, NFκB, XBP-1, PUR, and MAZ) that were downregulated in cells treated with ApoE2 + Aβ as compared to the cells treated with ApoE3 + Aβ. EMSA validations were performed for NFκB (which was 6.29×10^{-3} in the ApoE2 + Aβ treatment relative to ApoE3 + Aβ (Table 3)) and VDR (which was increased by ∼ 70x in ApoE2 + Aβ treatment relative to ApoE3 + Aβ (Table 3). The NFκB EMSA confirmed that the signalling pathway was activated by $A\beta_{1\text{-}42}$ as compared to Aβ scrambled, and that the ApoE2 + Aβ treatment resulted in significantly reduced DNA binding than Aβ alone or ApoE3 + Aβ treatment

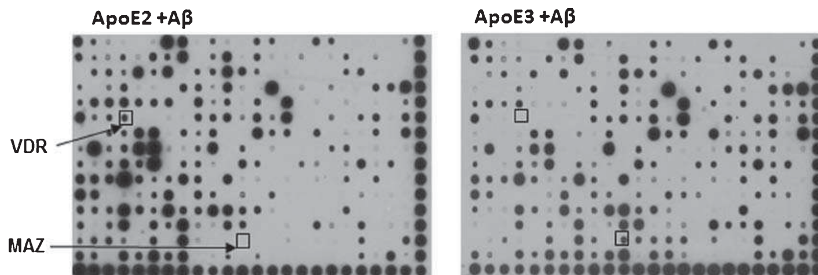

Fig. 5. Protein/DNA arrays to identify TFs activated in rat astrocytes treated with ApoE isoforms and $A\beta_{42}$. Protein/DNA array blots from cells treated with ApoE2 + Aβ (left panel) or ApoE3 + Aβ (right panel) are shown. Densitometry analysis of the arrays yielded 7 spots that were upregulated in ApoE2 + Aβ treatment (at-least 2-fold change in intensity compared to ApoE3 + Aβ) (Table 2), and thirty-six spots yielded the inverse relationship (downregulation in ApoE2 + Aβ as compared to ApoE3 + Aβ) (Table 2). VDR and MAZ were highlighted.

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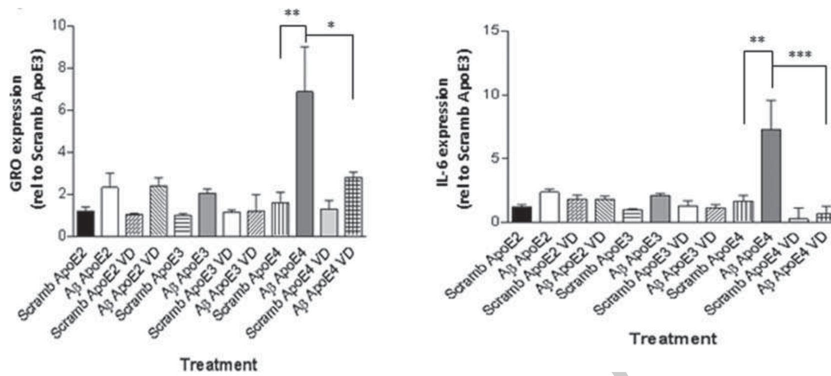

Fig. 6. Effect of VDR agonist 1α, 25-Dihydroxyvitamin D_3 on the expression of inflammatory genes in mouse apoE knock-out, human ApoE knock-in astrocytes upon challenge with $A\beta_{1-42}$. Mouse astrocytes expressing ApoE isoforms were treated with 0.1 μM 1α, 25-dihydroxyvitamin D_3. Expression of GRO (A) and IL-6 (B) were measured by RT-qPCR, normalized to GAPDH and presented (One-way ANOVA, Bonferroni *post-hoc*, $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$, $n = 3$).

(data not shown). The VDR EMSA results also agreed with the array observations. Aβ treatment showed significantly lower VDR binding than the scrambled control; while there was not a significant difference between ApoE2 + Aβ and ApoE3 + Aβ treatments, there was a clear trend that agreed with the array results (data not shown). These data show that the TF hits identified from the arrays highlight differential cellular pathways in the presence of ApoE isoforms and Aβ, and are potential candidates of interest in AD inflammation.

The effects of signaling modulation on Aβ-induced inflammatory response

To test if VDR signaling affects the expression of inflammatory genes in astrocytes, a VDR agonist 1α, 25-Dihydroxyvitamin D_3 (0.1 μM) was applied for 24 h to mouse astrocytes expressing human ApoE isoforms. Activation of VDR caused a significant reduction of the Aβ-induced upregulation of GRO and IL-6 observed in the ApoE4-expressing cell line (Fig. 6, One-way ANOVA, Bonferoni *post-hoc* test, $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$). There was no significant difference in either marker upon 1α, 25-Dihydroxyvitamin D_3 treatment of ApoE2- or ApoE3-expressing lines in the presence of Aβ or scrambled Aβ (Fig. 6). The expression of VDR in ApoE4/AD or ApoE2/AD mouse brains was not detected by immunoblot analysis (data not shown). BioGPS expression profile for mouse VDR shows that VDR expression is low in the brain and the antibodies we used may not be sensitive enough to detect the signal. The results suggest that inhibiting VDR signaling may be one of the main mechanisms of ApoE4-mediated promotion of Aβ-induced inflammatory response. Enhancement of VDR signaling by agonist can suppress ApoE4-promoted inflammatory response induced by $A\beta_{1-42}$ in astrocytes.

DISCUSSION

A number of studies have shown that ApoE isoforms have differential effects on Aβ aggregation, degradation and clearance and may affect synaptic function [8, 25–30]. However, little is known whether ApoE isoforms differentially regulate AD/Aβ-induced neuroinflammation and what mechanisms might be involved. Our study is the first to show that ApoE4 promotes AD/Aβ-induced inflammation while ApoE2 inhibits it. The roles of ApoE isoforms in AD neuroinflammation may be one of the mechanisms by which ApoE2 offers protection but ApoE4 promotes AD pathology. The presence of ApoE4 in AD mice increased brain inflammatory response compared to the ApoE2/AD mice. We also noticed that the level of $A\beta_{1-42}$ was higher in ApoE4/AD than in ApoE2/AD mice. Higher level of $A\beta_{1-42}$ may induce stronger inflammatory response, but this cannot explain the observations that $A\beta_{1-42}$ evoked significantly stronger inflammatory response in cultured ApoE4-expressing astrocytes than that in ApoE2- or ApoE3-expressing astrocytes. Furthermore, recombinant ApoE2 significantly inhibited $A\beta_{1-42}$-induced expression of inflammatory genes in cultured rat astrocytes as compared to recombinant ApoE3 or ApoE4. The combination of *in vitro* and *in vivo* data supports the notion that ApoE isoforms play differential roles in AD/Aβ-induced inflammatory response.

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One intriguing question is why A$\beta_{1\text{-}42}$ induced significantly stronger inflammatory response in ApoE4-expressing mouse astrocytes as compared to Aβ-treated ApoE3-or ApoE2-expressing cells, while recombinant ApoE4 + Aβ treatment did not promote a stronger inflammatory response compared to Aβ alone or recombinant ApoE3 + Aβ treatment in NRA cells. It has been shown that hApoE expression in immortalized murine astrocytes results in lipidated ApoE particles [22]. This suggests that lipidation state may affect ApoE4's role [22]. Thus, lipidated ApoE4 may promote stronger inflammatory response than ApoE2- or ApoE3-expressing cells treated with Aβ, whereas lipid-poor ApoE4 in NRA cells does not exacerbate the Aβ-induced inflammation relative to incubation with recombinant (lipid-poor) ApoE3. It should be noted from our *in vitro* and *in vivo* results that both lipid-poor and lipidated ApoE2 provided protection against A$\beta_{1\text{-}42}$-induced inflammatory gene expression. In rat astrocytes, lipid-poor ApoE2 inhibited Aβ-induced inflammatory gene expression as compared to astrocytes treated with Aβ alone or with Aβ + recombinant ApoE3 or ApoE4. Although inflammatory gene expression induced by A$\beta_{1\text{-}42}$ was higher than that induced by scrambled Aβ in ApoE2-expressing cells, there is no significant statistical difference between them, and furthermore, the expression of inflammatory genes induced by A$\beta_{1\text{-}42}$ was significantly lower in ApoE2-expressing cells than that in ApoE4-expressing cells. More importantly, ApoE2/AD mice had significantly lower levels of inflammatory cytokines than those in ApoE4/AD mice. This further endorses the notion that both lipid-poor and lipidated forms of ApoE2 offers protection against Aβ-induced inflammatory response. This may also explain why ApoE2 provides a greater degree of protection than ApoE3. Lipidated ApoE produced in mouse astrocytes is similar in structure to that seen in human brain, though not identical [22]. Delipidation of ApoE, as seen in ABCA1-knockout mice, promoted the formation of Aβ plaques in AD mice [31–33], while ABCA1 upregulation shows the opposite effect, decreasing Aβ levels [34]. The difference in ApoE4's pro-inflammatory activity between the two cell model systems may depend on ApoE's lipidation state, and suggests that further study into the role of lipidation in the inflammation seen in AD is warranted. Since both lipid-poor and lipidated ApoE proteins are present in human cells and tissues, the observed effects of lipid-poor and lipidated ApoE proteins could both be present in the brain.

One of the objectives of this study was to identify signaling pathways of importance in ApoE/Aβ inflammatory response. While some of the identified pathways are quite well-known (such as JNK-AP1 and NFκB pathways), many have not been substantially studied in the AD context. Vitamin D (VD) is a steroid hormone, primarily acting through VDR. VDR is a 427-amino acid protein belonging to the nuclear factor family of transcription factors. Several studies have found that polymorphisms in VDR gene are associated with risk of AD [35–37], further implicating this pathway in AD pathology. VDR is located in cytoplasm, and upon activation by active form of VD_3, VDR forms a heterodimer with RXR, translocates into nucleus, and binds to the vitamin D response elements (VDRE) on DNA to regulate target gene expression [38]. Interestingly, both VDR and RXR were found to be highly activated in ApoE2 + Aβ treated rat astrocytes on the Protein/DNA array, relative to ApoE3 + Aβ (Table 3). Studies have shown that VD is an important regulator of immune and inflammatory response. Zhang et al. reported that VD inhibits production of inflammatory cytokines by monocytes/macrophages and inhibits inflammatory response [39, 40]. VDR signaling can attenuate Toll-like receptor mediated inflammation [41]. *In vitro* studies have shown that VD protects neurons from Aβ-induced cytotoxicity and apoptosis [42], and VD/VDR signaling suppress AβPP production in cells [43]. Our study shows that active form of VD_3 almost completely inhibited inflammatory gene expression in ApoE4-expressing cells following Aβ treatment in comparison to the cells treated with A$\beta_{1\text{-}42}$. This suggests that VDR may be one of the major signaling pathways regulating AD/Aβ-induced inflammatory response. Interestingly, VD_3 did not affect Aβ-induced expression of inflammatory genes in ApoE3- or ApoE2-expressing cells. Since ApoE4-expressing cell line showed much higher inflammatory response to Aβ than the other cell lines, it is possible that VDR's effect was only observable in these highly inflammatory conditions. On the other hand, VDR might have already been activated in the presence of lipidated ApoE2 or ApoE3 with A$\beta_{1\text{-}42}$ since inflammatory gene expression had been inhibited in the absence of active form of VD_3. This is also supported by our observation that both VDR and RXR were highly activated in rat astrocytes pre-treated with lipid-poor recombinant ApoE2 for 24 h followed by treatment with A$\beta_{1\text{-}42}$. It is known that many VDR targets have neuroprotective and anti-inflammatory effects, including within

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astrocytes [44]. Thus, active form of VD_3 may be a preventative and/or therapeutic agent to relieve AD/Aβ-induced inflammatory response for reduced neuronal dysfunction and neurodegeneration, especially in ApoE4-carrier AD patients. VDR activation could be a potential AD therapeutic approach as a part of a wider attempt to affect a number of inflammatory pathways.

A clinical study [45] found that treatment of AD patients with memantine plus VD improved cognition performance. Annweiler and colleagues conducted a meta-analysis for 9 published studies and found that AD patients had lower levels of serum 25-hydroxylvitamin D_3 than matched controls [46], but the ApoE genotypes of the AD patients were not investigated in the analyses. Huebbe et al. found that ApoE4 carriers were associated with higher 25-hydorxylvitamin D_3 levels than ApoE2 and ApoE3 carriers in animal models and in normal human subjects [47]. There is no report in the literature of whether ApoE4/AD patients display lower or higher 25-hydorxylvitamin D_3 levels than age-matched non-demented controls or ApoE3/AD patients. 25-hydroxylvitamin D_3 is not an active form of VD and does not activate VDR signaling unless it is converted to 1,25-hydroxylvitamin D_3. It is likely that ApoE4 transgenic mice or ApoE4/AD patients may be less efficient in synthesizing active 1,25-dihydroxylvitamin D_3 from 25-hydroxylvitamin D_3, which remains to be investigated.

In summary, our studies have shown that ApoE isoforms play differential roles in regulating AD/Aβ-induced inflammatory response. ApoE4 seems to promote Aβ-induced inflammatory response while ApoE2 protects against it. *In vivo* experiment shows that ApoE4/AD mice had significantly higher levels of inflammatory cytokines than ApoE2/AD mice. *In vitro* studies reveal that lipidation states of ApoE isoforms may affect their roles in Aβ-induced inflammatory response. Lipidated ApoE4 enhanced $A\beta_{42}$-induced inflammatory response in astrocytes but not the recombinant lipid-poor ApoE4. Interestingly, lipidated ApoE3 provided a certain degree of protection while lipid-poor ApoE3 did not. Both lipidated and recombinant ApoE2 offered protection against $A\beta_{42}$-induced inflammatory response in astrocytes. VDR signaling was strongly activated in astrocytes pre-treated with recombinant ApoE2. Application of a VDR agonist, 1α, 25-dihydroxylvitamin D_3, almost completely inhibited $A\beta_{42}$-induced inflammatory gene expression in ApoE4-expressing cells. This suggests that VDR may be a major signaling pathway by which ApoE isoforms (lipidated and lipid-poor forms) play their differential roles in regulating ADs/Aβ-induced neuroinflammation. Active form of VD_3 or targeting VDR signaling may relieve the neuroinflammation and neurodegeneration seen in AD.

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