

ApoE genotype and an apoE-mimetic peptide modify the systemic and CNS inflammatory response

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Summary

Human apolipoprotein E is the major apolipoprotein expressed in the brain and exists as three isoforms, designated E2, E3, and E4. Although evidence suggests that apolipoprotein E plays an important role in modifying systemic and brain inflammatory responses, there is little data investigating apoE isoform-specific effects *in vivo*. In this study, we compared the inflammatory responses of targeted-replacement mice expressing the human apoE3 and apoE4 genes following intravenous administration of lipopolysaccharide. Animals expressing the E4 allele had significantly greater systemic and brain elevations of the pro-inflammatory cytokines TNF α and IL-6 as compared to their E3 counterparts, suggesting an isoform-specific effect of the immunomodulatory properties of apoE. Furthermore, intravenous administration of a small apoE-mimetic peptide derived from the receptor-binding region of the apoE holoprotein (apoE133-149) similarly suppressed both systemic and brain inflammatory responses in mice following lipopolysaccharide administration. These results suggest that apoE plays an isoform-specific role in mediating the systemic and brain inflammatory responses. Moreover, since exogenous administration of this apoE mimetic peptide is effective at suppressing both systemic and brain inflammation, it may represent a novel therapeutic strategy for diseases characterized by systemic or CNS inflammation, such as septic shock, multiple sclerosis, and traumatic brain injury.

Introduction

Apolipoprotein E (apoE protein; APOE gene) is a 34 kD protein originally studied for its role in cholesterol metabolism. There are three common human isoforms of apoE, designated E2, E3, and E4, encoded for by distinct alleles on human chromosome 19. The isoforms differ by single amino acid interchanges at residues 112 and 158: E3(Cys₁₁₂Arg₁₅₈), E4 (Arg₁₁₂Arg₁₅₈), and E2(Cys₁₁₂Cys₁₅₈). (1) The allele frequency of APOE4 is approximately 14%, and has been associated with the early onset of Alzheimer's disease (AD) and poor prognosis in neuroinflammatory disorders such as multiple sclerosis (2,3). The presence of the APOE4 allele is also associated with poor prognosis following traumatic brain injury (4,5) and with an increase in the systemic inflammatory response in patients following cardiopulmonary bypass (6).

ApoE is the major apolipoprotein produced in the central nervous system (CNS). Independent of its role in cholesterol metabolism, apoE modulates innate and acquired immune responses *in vitro* and *in vivo* (7). ApoE deficient animals have impaired immunity following bacterial challenge with *Listeria monocytogenes* (8). They also have increased susceptibility to endotoxemia after intravenous lipopolysaccharide (LPS) administration and following inoculation with *Klebsiella pneumonia* (9). A recent report confirmed the observation that apoE-deficient animals had an increased systemic inflammatory response and higher mortality following LPS injection, and that the administration of exogenous apoE improved mortality by downregulating the inflammatory cascade (10). The immunomodulatory properties of apoE may be of particular relevance in the CNS, where the absence of endogenous apoE has been shown to exacerbate cerebral edema and functional deficit following traumatic brain injury (11,12).

The mechanisms by which apoE exerts these immunomodulatory effects remain incompletely understood. Recent evidence suggests that, in addition to its role in lipid transport, apoE is capable of binding high affinity receptors and initiating a calcium dependent signaling response in immunocompetent cells (13). Specifically, the lipoprotein receptor related protein/ a 2 macroglobulin (LRP/a2M) receptor is capable of initiating signal transduction and modulating immune responses (14).

Although not extensively studied, isoform-specific differences in immune regulation may also play a particularly important role in mediating the CNS response to injury. For example, apoE4 is less effective than apoE3 or apoE2 at suppressing the activation of microglia in cell culture paradigms of brain inflammation (15, 16). In humans, isoform-specific differences are suggested by autopsy reports demonstrating increased numbers of scattered microglia and microglial activation in Alzheimer's disease (AD) patients carrying the APOE4 allele (17).

Although there is increasing evidence that apoE plays a biologically relevant role in modulating immune responses, the mechanism(s) by which this occurs remain unclear. It has been postulated that the protective effect of lipoproteins in endotoxemia is mediated by the binding and redirection of LPS from Kupffer cells to parenchymal liver cells, where endotoxin is inactivated and secreted into bile (18-20). An alternative hypothesis is that, independent of lipid binding, apoE downregulates activation of immune cells by binding to high affinity receptors and initiating a signaling cascade (21).

To investigate the mechanism by which apoE exerts its immunomodulatory effects, we looked at the ability of an apoE mimetic peptide to suppress the systemic and CNS inflammatory responses *in vivo*. This peptide is derived from the receptor binding region of apoE and is comprised of amino acid residues 133-149 (15). We have previously shown that apoE(133-149) retains the bioactivity of the intact apoE holoprotein in its ability to suppress brain macrophage activation and initiate a macrophage signaling cascade in cultured cells (13). We utilized an LPS model of induced inflammation in targeted replacement (TR) mice expressing the human E3 and E4 genes to establish whether systemic and brain inflammatory responses were influenced by apoE in an isoform-specific manner. These animals have the human apoE3 or E4 genes at the correct murine genetic locus and do not express murine apoE (20). The inflammatory response was monitored by measuring the temporal secretion and expression profiles of two pro-inflammatory cytokines, TNF α and IL-6, in the CNS and peripheral circulation. In this series of experiments, wildtype C57-BL6 mice were treated with a single intravenous injection of the apoE-mimetic peptide following LPS administration with TNF α and IL-6 levels determined. TNF α and IL-6 were selected to monitor the systemic and CNS immune response because they are both released from immune cells in the periphery during inflammation, are expressed in many CNS cells including microglia, neurons and astrocytes. In addition, TNF α and IL-6 play an important role in neuronal cell death and survival during injury and pathological conditions (22).

Experimental Procedures

Mice: APOE Target Replacement (TR) model was created by gene targeting of E14TG2a ES cells from 129P2/OlaHsd mice with the human apoE3 or human apoE4 construct in combination with flanking sequences from the 129 mouse. The targeted ES cells were injected into C57BL/6 blastocysts and the resulting chimeras were bred to C57BL/6 mice. They then underwent eight generations of backcrossing to C57BL/6. The colony is maintained by homozygous matings. Genotype is confirmed prior to each experiment. In addition, age-matched male C57-BL/6 mice were used in the peptide experiments.

Peptide Synthesis: Peptides were synthesized from the Peptide Synthesis Laboratory at the University of North Carolina at Chapel Hill (Chapel Hill, NC), to a purity of 95%, and were reconstituted in sterile isotonic PBS. For each peptide, the amino terminus was acetylated, and the carboxy terminus was blocked with an amide moiety. The 17 amino acid peptide was derived from apoE residues 133-149 (the receptor binding region):

Ac-LRVRLASHLRKLRKRL-amide. Control injections utilized the identical vehicle of PBS. To rule out the possibility of non-specific peptide effects, controls included a scrambled peptide of identical size and amino acid composition.

LPS and peptide injections: This study was approved by the Duke University Animal and Care Use Committee. 14-16 week old male C57-BL6 and matched APOE3-TR and APOE4-TR mice were injected with LPS (4g/kg in 100 μ L of PBS) via tail vein, and then immediately with vehicle (100 μ L of isotonic sterile PBS) or peptide (apoE133-149) (6.0 mg/kg, prepared in isotonic saline). Serum samples were obtained in LPS + vehicle and LPS + peptide groups (n=20 animals/group) at the following timepoints: baseline and at 1 hour, 3 hours and 24 hours after injection. Mice were anesthetized with isoflurane anesthesia and ventilated with 21% Oxygen. Blood was collected by transcardiac puncture, and allowed to clot for 30 minutes. Serum samples were centrifuged at 16,000 g for 5 minutes and quick-frozen by immersing in

liquid nitrogen and stored at -80°C . In order to remove systemic blood from the cerebral vasculature, mice were perfused with 20 mL of PBS via transcardiac puncture. Brains were removed and flash frozen in liquid nitrogen and stored at -80°C .

Quantification of cytokine protein: Cytokine levels in murine serum and brain homogenate were determined using Mouse Cytokine ELISA Kits for murine interleukin-6 (IL-6) and tissue necrosis factor alpha (TNF α) following the manufacturer's specifications (Pierce Endogen, Rockford, IL). Murine brains were isolated and quick-frozen by immersing in liquid nitrogen. The frozen brains were ground up into a fine powder in a liquid nitrogen pre-cooled mortar. Homogenates were generated by placing brains in ice-cold homogenized buffer (0.25 M sucrose, 1 mM EDTA, 10 mM HEPES [pH 7.4], 0.1% ethanol, and CompleteTM protease inhibitor Cocktail Tablets [Boehringer Mannheim, Heidelberg, Germany]) and homogenizing using a Teflon pestle and a motor-driven tissue homogenizer. Samples were maintained on ice throughout the homogenization procedure. Following homogenization, the sample was clarified by centrifuging at 5°C for 15 minutes at 1,500 g to remove cellular debris. The supernatant was removed and divided into several smaller working aliquots and stored at -70°C until ELISA analysis.

Quantification of mRNA: Murine brain total RNA was isolated by using Trizol reagent (Invitrogen Corporation, Carlsbad, CA) according to the manual. Cytokine gene expression will be determined by relative quantitative RT-PCR using Gene Specific Relative RT-PCR Kits (Ambion, USA) following the manufacturer's protocol. This method provides reagents, controls and methodology to yield reliable, relevant information about the relative abundance of different mRNA species in different RNA samples. It uses a multiplex RT-PCR approach in which two primer sets were used in a single reaction: one set to amplify the cDNA of interest, and a second to amplify an invariant endogenous control (18S rRNA). In addition to primers for the test gene and 18S PCR primer pair, a set of 18S PCR competitors are included to prevent loss of relative quantification. The PCR products were run on 6% acrylamide gels and visualized using a Molecular Dynamics PhosphorImager and Image Quant 5.0 software. For each sample, the signal obtained for the gene specific amplicon was divided by the signal obtained for the 18S amplicon yielding a corrected relative value for the gene specific product in each sample.

Statistical Analysis: Statistical differences in cytokine mRNA and protein were determined using 2-way ANOVA when data was parametric, and Wilcoxon rank test for nonparametric data.

Results

Effect of apoE isoform on inflammatory response:

To determine whether apoE modified systemic inflammatory responses in an isoform-specific manner, APOE3-TR and APOE4-TR animals were injected with LPS via the tail vein. Serum cytokine levels for TNF α and IL-6 were determined by ELISA at several intervals up to three hours post- injection.

Administration of LPS resulted in a strong induction of serum TNF α protein levels in both groups of transgenic animals. At one hour post-injection, serum TNF α was significantly higher in APOE4-TR animals compared to APOE3-TR animals (Figure 1A). At three hours, there was no measurable TNF α protein in either group (Figure 1A). Similarly, the same groups of animals exhibited a strong induction of serum IL-6 following injection of LPS (Figure 1B). IL-6 peaked at three hours in both groups, yet was significantly higher in the APOE4-TR animals relative to the APOE3-TR mice. The observation that animals bearing the human APOE3 gene had reduced serum cytokine levels for two markers of inflammation suggests that apoE3 is a more potent anti-inflammatory protein in a murine model of LPS-induced inflammation compared to apoE4.

We next determined whether endogenous apoE could similarly modify the primary brain inflammatory response in an isoform-specific manner. Peak brain TNF α RNA occurred one hour after the peripheral LPS injection, and was significantly higher in APOE4-TR animals compared to APOE3-TR animals. TNF α RNA was still elevated above baseline at three hours, but there were no significant differences at this time point (Figure 2A). Brain IL-6 mRNA was also elevated at one hour post-injection, and peaked at three hours. APOE4 animals had significantly higher levels at 1 hour, and no significant group differences were observed at three hours (Figure 2B). At 24 hours, both TNF α and IL-6 RNA were below the limits of sensitivity of this assay. Taken together, these results suggest an isoform-specific differential effect of apoE on modulating systemic and CNS inflammatory responses, with endogenous E4 less effective than E3.

We next investigated whether apoE (133-149), a peptide fragment derived from the receptor binding region of apoE would downregulate inflammatory responses in the same fashion as the intact apoE. When co-administered with LPS, the apoE-mimetic peptide significantly reduced serum TNF α levels at one hour, and reduced serum IL-6 levels at one

and three hours in wildtype C57-BL6 mice (Figure 3). There was no measurable TNF α or IL-6 protein at 24 hours post-injection in either group.

The anti-inflammatory effects of the peptide were similar in the brain, where the single intravenous injection of peptide significantly reduced TNF α and IL-6 RNA expression three hours after LPS injection (Figures 4A and 4B). The cytokine protein levels in the brain paralleled the RNA expression, and both TNF α and IL-6 protein were reduced following peptide injection (Figure 5).

To rule out the possibility that the downregulation of inflammatory cytokines in the CNS and systemic circulation were a non-specific peptide effect, we repeated these experiments using the scrambled apoE(133-149) sequence co-administered with LPS. We observed no differences in serum IL-6 between the scrambled peptide and vehicle group in serum IL-6 at 1 (18.1 ± 5.3 vs 21.5 ± 6.9 ng/ml) or 3 (56.4 ± 15.3 vs 52.9 ± 3.7 ng/ml) hours. Similarly, we found no significant difference in serum TNF α between the scrambled control and vehicle groups (443 ± 191 vs 778 ± 225 pg/ml at one hour and 3.1 ± 3 vs 3.9 ± 2.8 pg/ml at three hours). There was no difference in brain IL-6 at three hours post-injection (9.8 ± 0.5 vs 9.3 ± 0.3 pg/ml in vehicle and scrambled peptide treated animals, respectively). Brain TNF α levels were also not significantly different scrambled peptide and vehicle (56 ± 6 vs 39.1 ± 4 pg/ml). These results demonstrate that the scrambled apoE peptide retained no immunomodulatory bioactivity, and would suggest specificity for the apoE(133-149) peptide derived from the apoE receptor binding region.

Discussion

ApoE has been demonstrated to exert *in vitro* immunomodulatory effects by a number of investigators (23-25). The current study indicates that apoE is a biologically relevant immunomodulatory protein that exerts isoform-specific effects. In particular, mice expressing the human APOE4 transgene had higher levels of the pro-inflammatory cytokines TNF α and IL-6 in blood following LPS injection. Although the interaction of apoE genotype on immune function has not been definitively proven clinically, these results are consistent with a preliminary report demonstrating that patients with the APOE4 allele had a more robust systemic inflammatory response following cardiopulmonary bypass (6). In addition, apoE appears to mediate nitric oxide release after injury in an isoform specific fashion (26, 27).

We also found that the APOE4-TR mice have a more robust central nervous system (CNS) inflammatory reaction than APOE3-TR mice following peripheral injection of LPS. Since all animals were perfused to remove systemic blood from the cerebral vasculature, the increase in inflammatory cytokine RNA that we observed likely represents a primary brain inflammatory response. These results are consistent with a growing body of literature implicating apoE in modulating glial activation and the CNS response to injury. In cell culture experiments, apoE downregulates glial activation and the subsequent release of inflammatory mediators. This effect is isoform-specific; apoE4 is less effective than apoE3 (15, 16). The possibility that apoE4 is associated with a partial loss of function is also consistent with prior data demonstrating that apoE deficient mice have a more robust systemic and CNS inflammatory response in response to a variety of stimuli (28, 29). It is plausible that the relative ineffectiveness of the apoE4 isoform at suppressing the brain inflammatory response may play an important role in clinical neurological diseases associated with neuroinflammation and glial activation. In fact, the presence of the APOE4 allele has been associated with poor clinical outcome in multiple sclerosis, head injury, and an increased susceptibility of developing Alzheimer's disease (2-5).

The traditional mechanism by which apoE is thought to protect from LPS-induced lethality is by binding and redirecting LPS from macrophage to hepatocytes, with subsequent biliary excretion (18-20). However, apoE directly downregulates brain macrophage activation following exposure to a variety of structurally diverse stimuli, including LPS, amyloid precursor protein, and polyinosinic acid (15, 16). This suggests that apoE may also exert direct immunomodulatory effects that are independent of LPS binding. Given the fact that apoE

initiates a signaling cascade in macrophage, another possible mechanism of protection from LPS toxicity is that apoE directly modulates inflammatory responses by specific receptor interactions. In particular, apoE binds a family of lipoprotein receptor-related proteins (LRP/ α 2M), which have immunomodulatory properties and are present on macrophage and microglia (13, 14). It has recently been demonstrated that both apoE and the apoE mimetic peptide used in the present study directly compete for the same high affinity receptor on macrophage, and initiate a signaling cascade associated with increased production of inositol triphosphate and mobilization of intracellular Ca^{2+} stores. This signaling cascade is inhibited by pretreatment with receptor-associated protein and Nf^{2+} , and it is mediated by a pertussis-toxin sensitive G protein. (13).

To further explore this hypothesis, we created a series of small peptides derived from the receptor binding region of apoE. Several of these peptides, such as apoE(133-149) retain a significant degree of the native helical structure of the receptor binding region in the holoprotein, directly compete with the intact apoE protein for macrophage receptor binding, and retain the ability of the intact protein to downregulate microglial activation (15). It is noteworthy that the apoE peptide used in this study does not include residues 112 or 158, which are associated with the common human polymorphisms. *In vivo*, there is minimal difference in the protein structure between the apoE isoforms, although the free cysteine on residue 112 allows E2 and E3 to form homodimers (1). Although this polymorphic region is distinct from the receptor binding region, it is possible that the conformational changes induced by these adjacent amino acid substitutions affect the receptor binding region and subsequent apoE-receptor interactions. For example, the substitution of a cysteine for an arginine at position 158 significantly reduces the ability of apoE2 to bind the LDL receptor, even though this residue lies outside the receptor binding region (1, 30).

Our results suggest that endogenous apoE plays an important role in modifying both systemic and brain inflammatory responses. These effects appear to be isoform-specific, with the apoE4 isoform being less effective than apoE3 at downregulating inflammatory cytokines in the brain and peripheral circulation. Moreover in this study, we found that an apoE-mimetic peptide suppressed the release of the inflammatory cytokines $TNF\alpha$ and IL-6 following LPS injection. These results strongly suggest that the apoE-mimetic peptide retains the anti-inflammatory bioactivity of the parent protein. Unlike the intact apoE protein, which consists of 299 amino acid residues and has minimal penetration into the CNS compartment, small

apoE-mimetic peptides can potentially be modified to optimize blood-brain barrier penetration. This has implications for the rational design of novel therapeutic strategies targeting diseases characterized by systemic or CNS inflammation, such as septic shock, multiple sclerosis, and traumatic brain injury.

Figure Legends

Figure 1. Inflammatory cytokines are differentially expressed in APOE3 vs. APOE4 Targeted Replacement (TR) mice: 14-16 week old male targeted replacement APOE3-TR and APOE4-TR mice were injected with a single intravenous dose of 4.0 mg/kg LPS, and samples obtained by at 1, and 3, and 24 hours (n=20/group) and evaluated for tissue necrosis factor alpha (TNF α) and interleukin-6 (IL-6) levels by ELISA. **A:** At one hour post-LPS injection, there was a significantly increased level of TNF α in the apoE4 animals as compared to the apoE3 animals (p<0.05). **B:** At three hours, there was a significant increase in IL-6 levels in the apoE4-TR animals compared to apoE3-TR animals (p<0.05). Each data point represents 20 animals and is presented as mean \pm S.E.M)

Figure 2. Inflammatory genes are differentially regulated in the brain of E3 vs E4 TR mice: Following injection of 4.0 mg/kg LPS, APOE3-TR and APOE4-TR mice were sacrificed and perfused with PBS. **A:** Brain TNF α RNA peaked at 1 hour post-injection. ApoE4-TR animals had significantly higher levels of TNF α RNA than the apoE3 mice at 1 hour (p<0.05). **B:** Brain Il-6 RNA peaked at 3 hours post-injection, and the apoE4 animals had significantly higher Il-6 RNA than the apoE3 animals at 1 hour (p<0.05). Each data point represents 20 animals and is presented as mean \pm S.E.M.

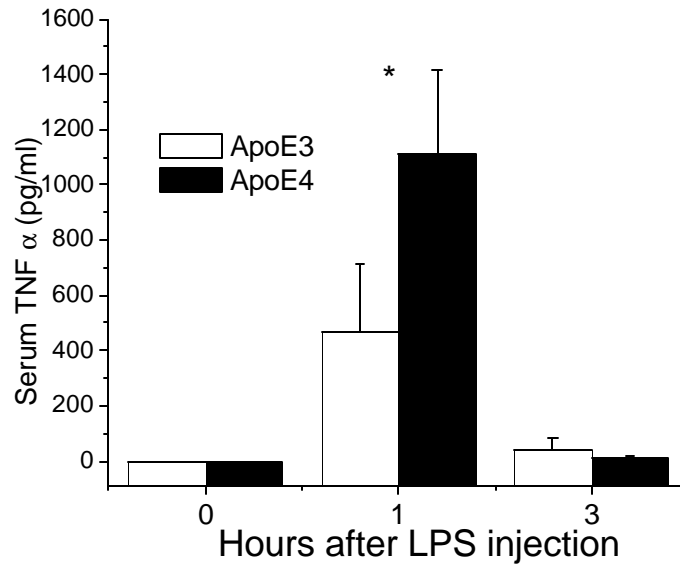
Figure 3. An apoE mimetic peptide significantly reduces serum cytokine levels after LPS stimulation: **A:** At 1 hour post-injection of LPS (4.0 mg/kg), serum TNF α was significantly reduced in animals receiving apoE(133-149), as compared to mice receiving vehicle (p<0.05). At 3 hrs and at baseline, TNF α levels were not measurable. **B:** At 1 and 3 hours post-injection, serum IL-6 levels were significantly reduced as a function of whether animals received LPS + vehicle or LPS + apoE(133-149) (p<0.05). At 24 hrs and at baseline IL-6 levels were not measurable. Each data point represents 8 animals and is presented as mean \pm S.E.M.

Figure 4. An apoE mimetic peptide significantly reduces brain cytokine RNA expression after LPS stimulation: **A:** Following injection of LPS (4.0 mg/kg), peak

brain TNF α RNA was demonstrated after three hours. Less TNF α RNA was present at three hours following LPS + apoE(133-149) compared to LPS + vehicle (P<0.05). **B:** Peak brain IL-6 RNA was also observed three hours after peripheral injection. Less TNF α RNA was present at three hours following LPS + apoE(133-149) compared to LPS + vehicle (p<0.05). Each data point represents 20 animals and is presented as mean \pm S.E.M.

Figure 5. An apoE mimetic peptide significantly reduces cytokine levels in the brain after LPS stimulation: Peak inflammatory cytokine protein levels in brain were present three hours after LPS injection (4.0 mg/kg). **A:** At one and three hours, there was a significant reduction in brain TNF α protein following LPS injection in animals treated with apoE(133-149) peptide compared to controls (p<0.05). **B:** At three hours, there was a significant reduction in brain IL-6 protein following LPS injection in animals treated with apoE(133-149) peptide compared to controls (p<0.05). Each data point represents 8 animals and is presented as mean \pm S.E.M.

A



B

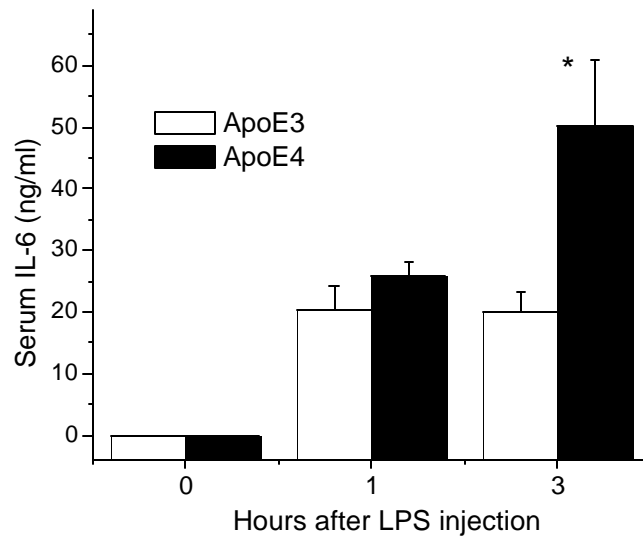
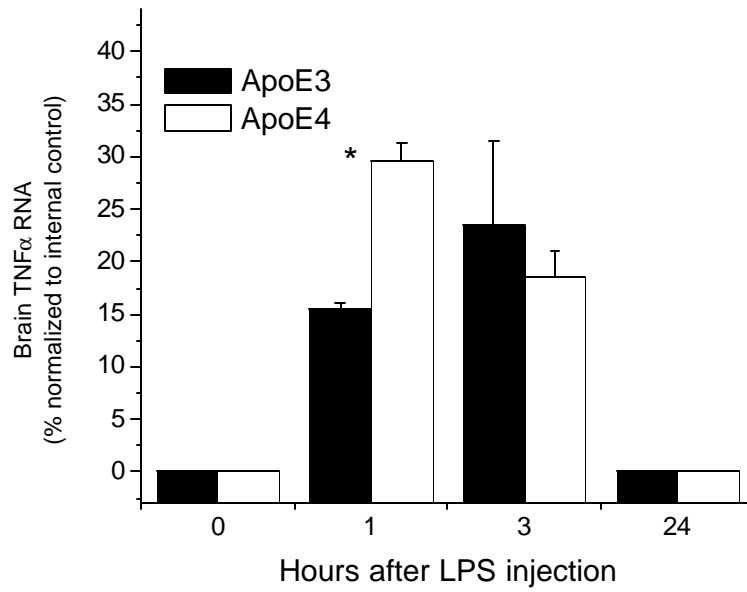


Figure 1

A



B

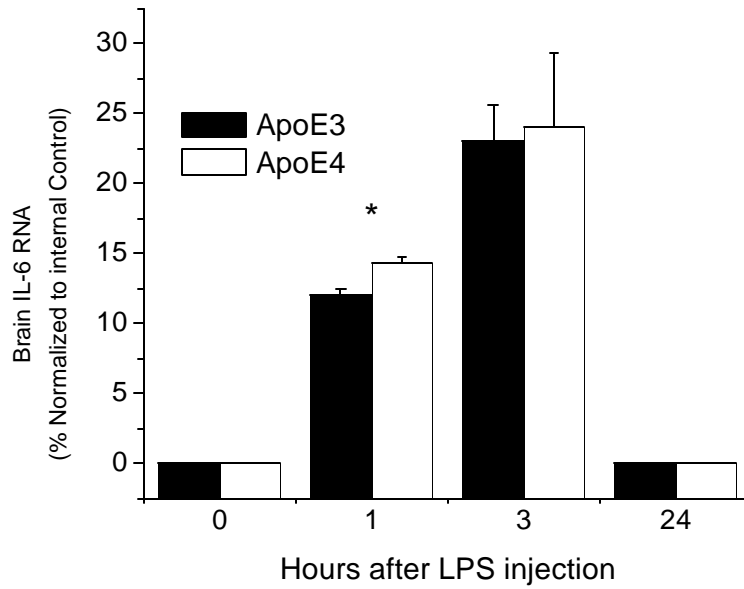
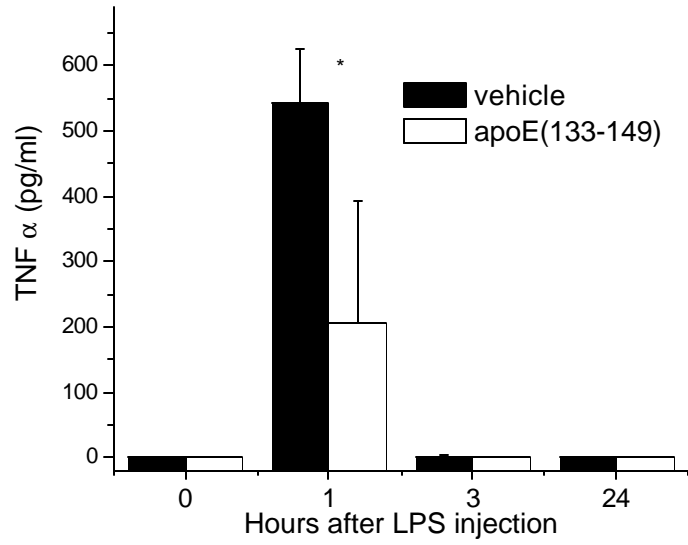


Figure 2

A



B

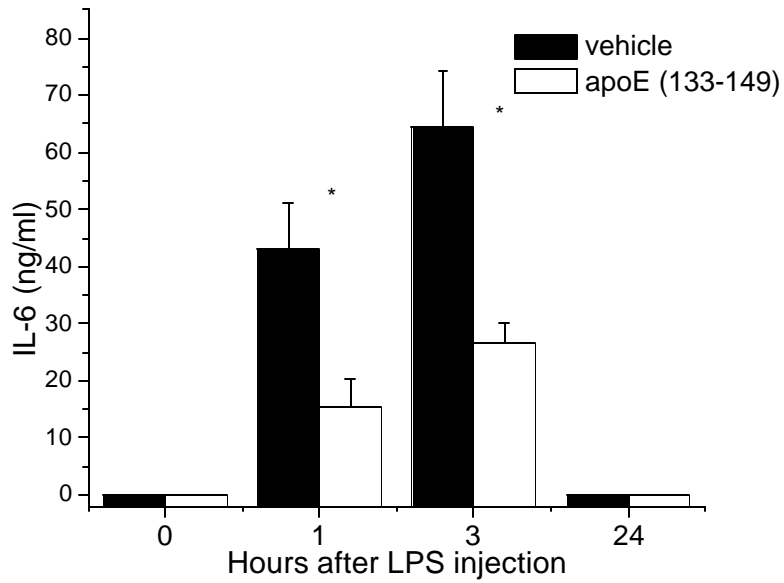
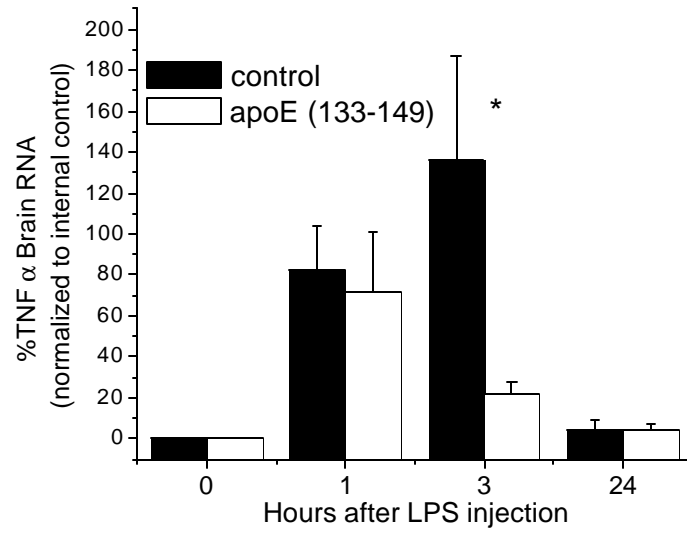


Figure 3

A



B

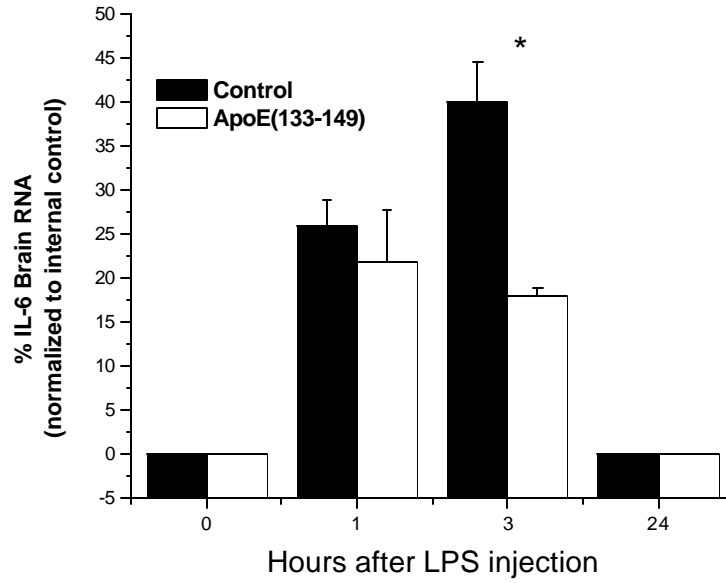
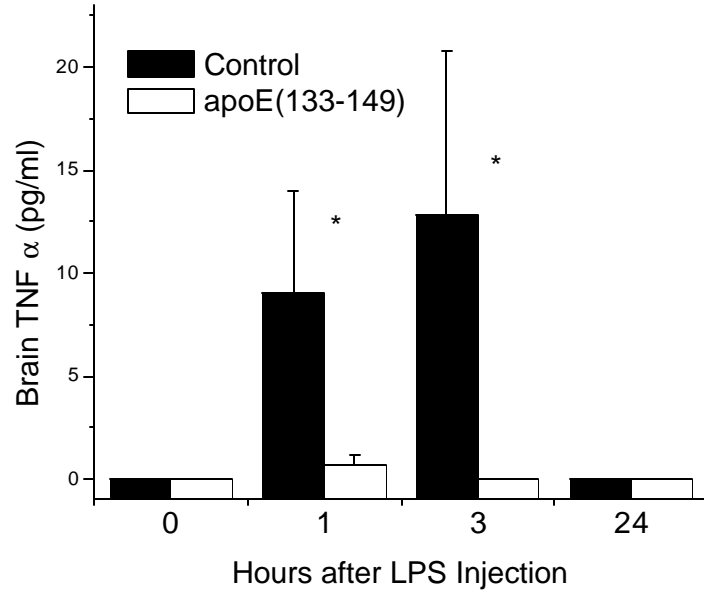


Figure 4

A



B

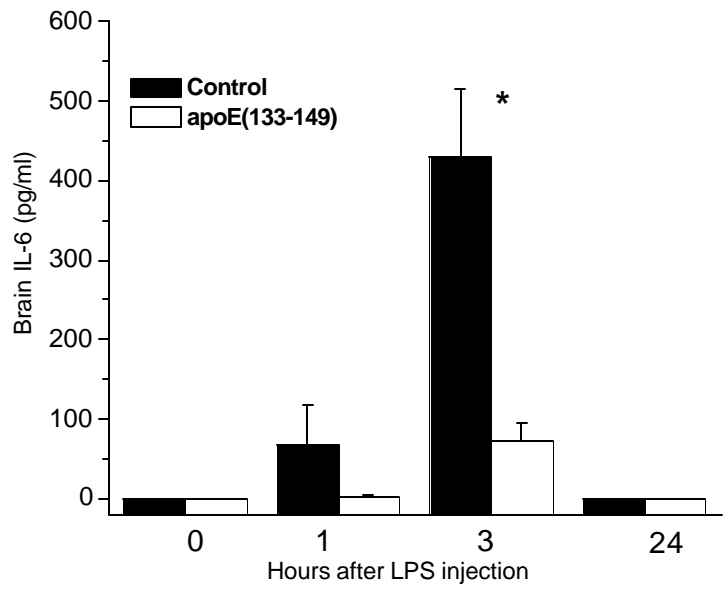


Figure 5

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