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 after intravenous administration of lipopolysaccharide. Animals expressing the E4 allele had significantly greater systemic and brain elevations of the pro-inflammatory cytokines TNFα and IL-6 as compared with their APOE3 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 (apoE-(133–149)) similarly suppressed both systemic and brain inflammatory responses in mice after lipopolysaccharide administration. These results suggest that apoE plays an isoform-specific role in mediating the systemic and brain inflammatory responses. Moreover, because 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 central nervous system inflammation, such as septic shock, multiple sclerosis, and traumatic brain injury.

Apolipoprotein E (apoE protein; APOE gene) is a 34-kDa 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(Cys112-Arg158), E4(Agi12-Arg158), and E2(Cys112-Cys158) (1). The allele frequency of APOE4 is 14% and has been associated with the early onset of Alzheimer’s disease 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). Independently 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 after bacterial challenge with Listeria monocytogenes (8). They also have increased susceptibility to endotoxemia after intravenous lipopolysaccharide (LPS) administration and inoculation with Klebsiella pneumoniae (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 down-regulating 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 after 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/α-2 macroglobulin receptor is capable of initiating signal transduction and modulating immune responses (14).

Although not extensively studied, isoform-specific differences in immune regulation may 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 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 hy-
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pothesis is that, independent of lipid binding, apoE down-regulates activation of immune cells by binding to high affinity receptors and initiating a signaling cascade (13). 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 composed of amino acid residues 133–149 (15). We have shown previously 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, TNFα and IL-6, in the central nervous system and peripheral circulation. In this series of experiments, wild-type C57BL6/J mice were treated with a single intravenous injection of the apoE-mimetic peptide after LPS administration with TNFα and IL-6 levels were determined. TNFα 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, TNFα and IL-6 play an important role in neuronal cell death and survival during injury and pathological conditions (22).

EXPERIMENTAL PROCEDURES

Mice—APOE TR model was created by gene targeting of E14Tg2a embryonic stem cells from 129P2/OlaHsd mice with the human apoE3 or human apoE4 construct in combination with flanking sequences from the 129 mouse. The targeted embryonic stem cells were injected into C57BL6/J blastocysts, and the resulting chimeras were bred to C57BL6/J mice. Then, they underwent eight generations of back-crossing to C57BL6/J. The colony was maintained by homozygous matings. Genotype was confirmed prior to each experiment. In addition, age-matched male C57BL6/J mice were used in the peptide experiments.

Peptide Synthesis—Peptides were synthesized from the Peptide Synthesis Laboratory at the University of North Carolina (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 carboxyl terminus was blocked with an amide moiety. The 17-amino acid peptide was derived from apoE residues 133–149 (the receptor-binding region): Ac-LRVRLASHLRKLRKRLL-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 C57BL6 and matched APOE3-TR and APOE4-TR mice were injected with LPS (40 μg/kg in 100 μl PBS) via tail vein puncture and allowed to clot for 30 min. Serum samples were centrifuged at 16,000 × g for 5 min, quick-frozen by immersing in liquid nitrogen, and stored at −80 °C. To remove systemic blood from the cerebral vasculature, mice were perfused with 20 ml of PBS via transcardiac puncture and allowed to clot for 30 min. Serum samples were centrifuged at 16,000 × g for 5 min, quick-frozen by immersing in liquid nitrogen, and stored at −80 °C. To remove systemic blood from the cerebral vasculature, mice were perfused with 20 ml of PBS via transcardiac puncture and allowed to clot for 30 min. Serum samples were centrifuged at 16,000 × g for 5 min, quick-frozen by immersing in liquid nitrogen, and stored at −80 °C.

Quantification of Cytokine Protein—Cytokine protein levels in murine serum and brain homogenate were determined by using mouse cytokine ELISA kits for murine IL-6 and TNFα 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 reverse transcription-PCR approach in which two primer sets were used in a single reaction: one set was used to amplify the cDNA of interest, and a second was used 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% acrylicamide gels and visualized using a PhosphorImager and Image Quant version 5.0 software (Molecular Dynamics). 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 by using 2-way ANOVA when data was parametric and a 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 TNFα and IL-6 were determined by ELISA at several intervals up to 3 h after injection.

Administration of LPS resulted in a strong induction of serum TNFα protein levels in both groups of transgenic animals. At 1 h after injection, serum TNFα was significantly higher in APOE4-TR animals compared with APOE3-TR animals (Fig. 1A). At 3 h, there was no measurable TNFα protein in either group (Fig. 1A). Similarly, the same groups of animals exhibited a strong induction of serum IL-6 after injection of LPS (Fig. 1B). IL-6 peaked at 3 h 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 with apoE4.

We next determined whether endogenous apoE could similarly modify the primary brain inflammatory response in an isoform-specific manner. Peak brain TNFα RNA occurred 1 h after the peripheral LPS injection and was significantly higher in APOE4-TR animals compared with APOE3-TR animals. TNFα RNA was still elevated above baseline at 3 h, but there were no significant differences at this time point (Fig. 2A). Brain IL-6 mRNA was also elevated at 1 h after injection, and peaked at 3 h. APOE4 animals had significantly higher levels at 1 h, and no significant group differences were observed at 3 h (Fig. 2B). At 24 h and at baseline, IL-6 levels were not measurable. Each point of the data represents eight animals and is presented as mean ± S.E.

Next, we investigated whether apoE-(133–149), a peptide fragment derived from the receptor-binding region of apoE, would down-regulate inflammatory responses in the same fashion as the intact apoE. When co-administered with LPS, the apoE-mimetic peptide significantly reduced serum TNFα levels at 1 h, and reduced serum IL-6 levels at 1 and 3 h in wild-type C57BL6/J mice (Fig. 3). There was no measurable TNFα or IL-6 protein at 24 h after 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 TNFα and IL-6 RNA expression 3 h after LPS injection (Figs. 4, A and B). The cytokine protein levels in the brain paralleled the RNA expression, and both
TNFα and IL-6 protein were reduced after peptide injection (Fig. 5).

To rule out the possibility that the down-regulation of inflammatory cytokines in the CNS and systemic circulation were a nonspecific 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 control and vehicle groups at 1 h (18.1 ± 5.3 versus 21.5 ± 6.9 ng/ml) or 3 h (56.4 ± 15.3 versus 52.9 ± 3.7 ng/ml). Similarly, we found no significant difference in serum TNFα between the scrambled control and vehicle group in serum IL-6 at 1 h (18.1 ± 5.3 versus 21.5 ± 6.9 ng/ml) or 3 h (56.4 ± 15.3 versus 52.9 ± 3.7 ng/ml). There was no difference in brain IL-6 at 3 h after injection (9.8 ± 0.5 versus 9.3 ± 0.3 pg/ml in vehicle and scrambled peptide treated animals, respectively). Brain TNFα levels were also not significantly different in scrambled peptide and vehicle (56 ± 6 versus 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 gene had higher levels of the pro-inflammatory cytokines TNFα and IL-6 in blood after 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 after cardiopulmonary bypass (6). In addition, apoE seems to mediate nitric oxide release after injury in an isoform-specific fashion (26, 27).
CNS inflammatory response than APOE3-TR mice following peripheral injection of LPS. Because 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 down-regulates 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 peripheral and CNS inflammatory response to a variety of stimuli (28, 29). It is plausible that the relative ineffectiveness of the apoE-mimetic peptide suppressed the release of the inflammatory cytokines TNFα and IL-6 after 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 possibility 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.

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APOE Genotype and an ApoE-mimetic Peptide Modify the Systemic and Central Nervous System Inflammatory Response
John R. Lynch, Wen Tang, Haichen Wang, Michael P. Vitek, Ellen R. Bennett, Patrick M. Sullivan, David S. Warner and Daniel T. Laskowitz

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