Apolipoprotein E Suppresses the Type I Inflammatory Response In Vivo

Kamilah Ali, Melissa Middleton, Ellen Puré, Daniel J. Rader

Abstract—Apolipoprotein E (apoE) is synthesized in the liver and in macrophages, and it has antiatherogenic properties that are mediated, at least in part, through the regulation of plasma cholesterol homeostasis. Previous data suggest that apoE also has antiinflammatory properties that may contribute to protection against atherosclerosis independent of its role in lipid metabolism. In this study, apoE knockout and C57BL/6 mice were stimulated with low-dose lipopolysaccharide (LPS) and other Toll-like receptor (TLR) agonists. We show that apoE modulates the systemic type I inflammatory response in vivo. The proinflammatory cytokines tumor necrosis factor α, interleukin (IL)-6, IL-12, and interferon-γ were upregulated to a significantly greater extent in apoE-deficient mice than in wild-type mice at both the mRNA and protein levels following administration of LPS. In contrast, hypercholesterolemic low-density lipoprotein receptor/apobec-1 double knockout mice had a similar cytokine response as wild-type mice, eliminating hypercholesterolemia as a cause for the exaggerated cytokine response. Importantly, reconstitution of apoE expression in the liver of apoE-deficient mice normalized the LPS-induced plasma protein levels of IL-12p40. Furthermore, there was selective upregulation of plasma IL-12 in apoE knockout mice by a TLR3 agonist, poly I:C, but not by other TLR agonists, CpG oligonucleotide or Toxoplasma gondii antigen. This implies that apoE selectively regulates TLR4- and TLR3-mediated signaling of IL-12 production. These results indicate that apoE modulates the T helper-1–type immune response in vivo by modulating IL-12 production. (Circ Res. 2005;97:922-927.)

Key Words: apolipoprotein E ■ inflammation ■ interleukin-12 ■ lipopolysaccharide ■ T helper-1

Apolipoprotein E (apoE) is a 34-kDa secreted protein synthesized in liver and macrophages that has been shown to be highly antiatherogenic. ApoE was first described with regard to its effects on plasma lipoprotein metabolism and cholesterol homeostasis, specifically its ability to facilitate clearance of triglyceride-rich lipoproteins in the liver. Indeed, at least part of its ability to protect against atherosclerosis is related to its effects on lipoprotein metabolism. However, evidence indicates that apoE has antiatherogenic properties independent of plasma lipoprotein regulation.1–3

Previous data support a role for apoE as an immunomodulatory agent. Initial observations in vitro demonstrated that apoE could inhibit lymphocyte proliferation.4 Furthermore, apoE inhibits proliferation of antigen and mitogen stimulated CD4+ and CD8+ T cells indirectly via reduction of interleukin (IL)-2.5 Other studies have focused on understanding the role of apoE in regulating acute inflammation. Infection of apoE knockout mice with Listeria monocytogenes or Klebsiella pneumoniae resulted in an increased susceptibility to death as well as increased serum levels of tumor necrosis factor (TNF-α) expression compared with wild-type mice.6–8 ApoE-deficient mice injected with the TLR4 agonist lipopolysaccharide (LPS) produced similarly exaggerated cytokine levels of TNF-α, IL-1β, and IL-6, and, in addition, exogenous apoE could reverse this effect in rats.9

Although these data provide evidence that apoE can affect both the innate and adaptive immune responses, none of the studies of apoE to date has assessed its ability to affect cytokine levels triggered by stimulation with other Toll-like receptors (TLRs), nor its ability to regulate the nature of the immune response. The balance between type I and type II immune responses is critical to determining the outcome of pathogen assault, as well as the development of atherosclerotic disease. The resulting cytokine milieu is based on the nature of the immune system, as well as the specific stimulus that has activated it. The type I response is characterized by the production of IL-12 and interferon (IFN)-γ. Each of these cytokines is essential for type I inflammatory responses and have been shown to promote the progression of atherosclerosis.9–13 Given that apoE protects against atherosclerosis, as well as the previous evidence showing the modulatory role of apoE on inflammation, we hypothesized that apoE may regulate the expression of IL-12 and IFN-γ, and thereby the type I response, during acute inflammation in a stimul-
selective manner. To test this hypothesis, we measured plasma levels of IL-12 and IFN-γ induced by LPS and other TLR agonists in apoE−/− compared with wild-type mice.

Material and Methods

Animal Experiments

Six- to 8-week-old female apoE−/− (before atherosclerotic lesion development) and C57BL/6 mice were obtained from Jackson Laboratory (Bar Harbor, Me) and Taconic (Germantown, NY) (n = 3 to 6 per group). LDLR/apobec double knockout mice were bred in house (n = 3 to 6). Mice were fed a chow diet and handled according to the guidelines of the University of Pennsylvania Institutional Animal Care and Use Committee. Mice received IP injections of a pyrogen-free PBS containing 12.5 µg of LPS 0111:B4 (LPS, Sigma), 100 ng of polyinosine-polycytidylic acid (poly I:C) (Amersham), 10 nmol of CpG 1826 oligonucleotide (Oligos Etc Inc), Toxoplasma gondii soluble parasite lysate antigen (courtesy of Dr Chris Hunter, University of Pennsylvania), or buffer alone. At various time intervals, mice were anesthetized or euthanized by IP administration of ketamine/xylazine, then bled retroorbitally or blood was collected from inferior vena cava. For extraction of tissues, the heart was perfused with cold PBS and organs were excised, and stored in RNAlater solution (Ambion) at 4°C. The aorta was removed from the aortic arch, most proximal to the heart, to the postbifurcation artery free of periadventitial fat.

To reconstitute hepatic apoE expression, apoE−/− mice were injected with an adeno-associated vector (AAV) consisting of serotype 2/8 encoding human apoE3 (AAV2/8.TBG.hapoE3) under the control of liver-specific promoter, thyroid binding globulin (TBG), or with control AAV2/8 encoding the lacZ gene (AAV2/8.TBG.lacZ) into the tail vein (100 g of polyinosine-polycytidylic acid (poly I:C) (Amersham), 10 nmol of CpG 1826 oligonucleotide (Oligos Etc Inc), Toxoplasma gondii soluble parasite lysate antigen (courtesy of Dr Chris Hunter, University of Pennsylvania), or buffer alone. At various time intervals, mice were anesthetized or euthanized by IP administration of ketamine/xylazine, then bled retroorbitally or blood was collected from inferior vena cava. For extraction of tissues, the heart was perfused with cold PBS and organs were excised, and stored in RNAlater solution (Ambion) at 4°C. The aorta was removed from the aortic arch, most proximal to the heart, to the postbifurcation artery free of periadventitial fat.

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Cytokine and Plasma Lipid Analysis

Mouse plasma levels of cytokines and inflammatory mediators were measured by ELISA according to the protocol of the manufacturer from the following sources: TNF-α, IFN-γ, IL-6 (R&D Systems and BD Bioscience); serum amyloid A (SAA) (BioSource International), and IL-12 (BD Bioscience). Plasma total cholesterol, high-density lipoprotein, triglyceride, and human apoE levels were measured using commercial assay kits (Wako Pure Chemical Industries Ltd).

Real-Time Polymerase Chain Reaction

Total RNA was isolated by homogenization of tissue and subsequent use of the RNeasy mini kit with on-column DNase treatment (Qiagen Inc). The concentration of total RNA was determined spectrophotometrically at 260 nm. cDNA was synthesized from 1.5 µg of total RNA by reverse transcription using Superscript II reverse transcriptase enzyme (Invitrogen). Primers for IL-12p35, IL-12p40, SAA, and β-actin were designed using the default settings of the manufacturer for the Primer Express 2.0 program (Applied Biosystems) or previously published sequences; IFN-γ and IL-6 (Table).15–16 mRNA levels were quantitatively determined on an ABI Prism 7000 sequence detection system (Applied Biosystems) using SYBR-green dye. The relative mRNA of each target gene was normalized to a housekeeping gene, β-actin, and then fold induction was determined above the levels of C57BL/6 PBS control group for each tissue.

Statistical Analysis

Results were analyzed by one way variance ANOVA or unpaired 2-tailed Student’s t test using Graphpad Prism 3.0 software (GraphPad). Values of P<0.05 were considered statistically significant.

Results

Elevated Levels of Proinflammatory Cytokines in ApoE−/− Mice After LPS Stimulation

To evaluate the in vivo effects of apoE during acute inflammation, the levels of proinflammatory cytokines produced in LPS-injected mice were analyzed at the time of the peak response for each cytokine, as determined in preliminary kinetic experiments (Figure 1A). Production of TNF-α and IL-6 in apoE−/− mice was significantly increased, which is consistent with previous observations with apoE−/− mice (Figure 1A). Strikingly, IL-12 production was also elevated in apoE−/− compared with wild-type mice at the level of the p40 chain as well as the biologically active p70 heterodimer (Figure 1A). Analysis at various times post LPS injection indicated that the difference in cytokine production was not merely a reflection of a shift in the kinetics of the response in apoE compared with C57BL/6 mice (Figure 1B). Furthermore, IFN-γ levels (only detectable at 6 hours) were significantly higher in apoE−/− mice than in wild-type mice (Figure 1B). In contrast, a nonspecific liver-derived inflammatory marker, SAA, was upregulated to a significantly lesser extent in apoE−/− mice compared with wild-type mice (Figure 1B). To assess whether the ability of apoE to modulate cytokine levels was dependent on its profound effects on lipoprotein metabolism, another hypercholesterolemic model, the LDLR/apobec double knockout mouse, was examined during LPS insult. In 2 independent experiments, TNF-α, IL-6, and IL-12 plasma levels were comparable on LPS stimulation between wild-type and LDLR/apobec double knockout mice (data not shown). The average total cholesterol levels of LDLR/apobec−/− mice were 395 mg/dL versus 589 mg/dL for apoE−/− mice. This suggests that the exacerbated cytokine...
production in apoE-deficient mice is independent of the induction of hypercholesterolemia.

**Differential Expression of Proinflammatory Cytokine Genes After LPS Stimulation**

To better understand the mechanism by which apoE regulates inflammation, the cytokine mRNA levels in apoE−/− and wild-type mice were examined in the liver and spleen after LPS stimulation. The mRNA levels of IL-6, IFN-γ, and IL-12p40 were significantly upregulated in 1 or both tissues examined and mirrored the differences in the plasma protein levels in apoE−/− mice compared with control wild-type mice (Figure 2). We also found that IL-12p40 expression was significantly higher in the aortae from apoE−/− compared with wild-type mice, although the absolute mRNA expression was relatively low compared with the other tissues (data not shown). The upregulation of IL-12 in the aorta of young mice before lesion most likely reflects adhesion of leukocytes such as macrophages to the LPS-activated endothelium of the arterial cell wall, as the macrophage mRNA marker, CD68, was increased in aortae of both wild-type and apoE−/− mice after LPS stimulation (data not shown). These results indicate that apoE regulates the expression of type I and other cytokines at the mRNA level.

**Effect of Reconstitution of Hepatic ApoE Expression on the Inflammatory Response in ApoE−/− Mice**

Because apoE is synthesized predominantly in the liver and in macrophages, and potentially by other cell types not yet reported, the effects in apoE-deficient mice could be attributable to lack of liver-derived circulating apoE and/or mediated by apoE produced by hematopoietic cells, such as macrophages. Indeed, reconstitution of apoE-deficient mice with apoE wild-type macrophages or bone marrow–derived cells can reduce the atherosclerosis associated with the apoE-deficient phenotype, indicating the importance of apoE in leukocyte regulation.1,17 To determine whether the observed increase in TNF-α, IL-6, IL-12, and IFN-γ expression was attributable to the absence of plasma apoE or specifically to lack of cellular apoE expression, a recombinant AAV vector encoding human apoE under the control of a liver-specific promoter was used to induce long-term expression of apoE. Plasma human apoE levels were detected in apoE−/− mice at an average of 5.6 mg/dL after injection with the human apoE AAV vector. After 17 to 21 days, LPS was injected IP into mice and serum plasma was collected. The kinetics of cytokine production was the same as depicted in Figure 1; however, only the optimal time of expression is shown in Figure 3. Importantly, IL-12 plasma levels after LPS injection in AAV.hapoE apoE−/− mice were reduced to levels detected in AAV.lacZ wild-type mice, whereas TNF-α levels were significantly reduced in AAV.hapoE apoE−/− mice but not completely restored to the levels detected in AAV.lacZ treated wild-type mice (Figure 3A and 3C). In contrast, the plasma levels of IL-6 and IFN-γ were not affected by the expression of hepatic human apoE (Figure 3B and 3D), although, there was a trend of reduction in IFN-γ plasma levels in AAV.hapoE apoE−/− mice compared with AAV.lacZ apoE−/− mice. This suggests that the levels of IL-12 and TNF-α were influenced by plasma apoE, whereas other sources of apoE, ie, macrophages, may directly modulate these other proinflammatory mediators. The overexpres-

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**Figure 1.** ApoE regulates the inflammatory response to LPS in vivo. A, Plasma levels of proinflammatory molecules in apoE knockout and wild-type mice were measured at the time of the peak response for each cytokine after IP injection of 12.5 μg of LPS: TNF-α at 1 hour, IL-6 at 3 hours, IL-12p40 at 3 hours, and IL-12p70 at 3 hours. B, The plasma levels of the indicated inflammatory mediators were measured at the indicated times postinjection of LPS: wild-type (cross-hatched bars) and apoE knockout (solid black bars) mice. The results represent the mean of 4 to 7 mice per group ± SEM. *P<0.05. n.d. indicates levels that are not detectable.
tion of human apoE in wild-type mice did not affect the LPS-induced response (data not shown), indicating that endogenous apoE levels in wild-type mice are adequate to optimally suppress the levels of these inflammatory cytokines (data not shown).

**ApoE Selectively Modulates IL-12 Production In Vivo**

To determine whether apoE selectively modulates TLR4-mediated induction of IL-12, wild-type and apoE<sup>−/−</sup> mice were stimulated with 3 different TLR agonists: *Toxoplasma gondii* soluble parasite lysate antigen (STAg) (TLR2/11), CpG DNA (TLR9), and poly I:C (TLR3). As shown in Figure 4A, STAg and CpG DNA induced similar levels of IL-12p40 between wild-type and apoE<sup>−/−</sup> mice, indicating that apoE regulation of IL-12 production is stimuli selective. In contrast, IL-12p40 levels were significantly elevated in apoE<sup>−/−</sup> mice compared with wild-type on stimulation with poly I:C, revealing a novel role for apoE in the TLR3-signaling pathway (Figure 4A). Further examination of the effect of poly I:C on the production of other cytokines at the times of their peak expression showed the poly I:C effect to be selective to IL-12, as no difference in the plasma expression levels of TNF-α, IL-6, or IFN-γ was observed between apoE<sup>−/−</sup> and wild-type mice (Figure 4B through 4D). Thus these results suggest that apoE selectively modulates TLR4- and TLR3-induced IL-12 production.

**Discussion**

In this study, apoE was found to regulate the LPS and poly I:C–induced type I inflammatory response in vivo. Mice lacking apoE had a substantially exaggerated response to LPS with regard to TNF-α and IL-6, as previously observed, but we also reveal, for the first time, that production of the critical T helper-1 cytokines, IL-12, and IFN-γ are also exaggerated in apoE-deficient mice. Reconstitution of plasma levels of apoE in apoE knockout mice normalized LPS-induced IL-12 and significantly reduced LPS-induced TNF-α plasma levels. Additional evidence indicated that the modulation of IL-12 was specifically the result of apoE deficiency and excluded hypercholesterolemia as a possible mechanism for the exaggerated cytokine levels in apoE<sup>−/−</sup> mice. Moreover, apoE appears to modulate IL-12 production in a selective manner by regulating both TLR4- and TLR3-mediated induction of IL-12 but not other TLR signaling pathways leading to IL-12 production. These findings provide the first link between apoE and IL-12 and IFN-γ production, revealing that apoE modulates a critical inflammatory axis in atherosclerotic disease.

The molecular mechanisms of the immunomodulatory effects of apoE in vivo are still unclear. Previous studies have shown that apoE may regulate LPS-induced cytokine production by binding LPS, redirecting it to bile, and thereby preventing it from binding to its receptor. However, this is unlikely to be the mechanism responsible for the effects reported here because the effect of apoE on LPS-induced genes appears to be selective, as levels of the liver-derived acute-phase protein SAA were not higher in the apoE<sup>−/−</sup> mice. Furthermore, the fact that overexpression of apoE in wild-type mice did not result in reduced cytokine levels in wild-type mice argues against this mechanism. Our data suggest that whereas poly I:C and LPS may stimulate IL-12 production via either similar or distinct pathways, these agonists induce the production of other proinflammatory cytokines, such as IL-6 and TNF-α, through different mechanisms, because apoE regulated IL-12 production in response to both agonists, whereas only LPS-induced production of TNF-α and IL-6 was regulated by apoE.

During an innate immune response, TLRs recognize specific pathogen-associated molecular patterns to induce gene expression. Ligand-dependent conformational changes of the
receptors results in TLR recruitment of adaptor molecules necessary for signal transduction. Most TLR signaling is at least partially dependent on the adaptor molecule myeloid differentiation primary-response protein 88 (MyD88). It is known that TLR4 and TLR3 can signal via both MyD88-dependent and -independent pathways. Although TLR3 signaling is thought to be mediated predominantly through a MyD88-independent pathway, the data is still controversial as to the MyD88 dependency of TLR3-induced cytokine production. However, it is currently unknown whether or not TLR3 includes IL-12 production in a MyD88-independent manner. If TLR3-mediated IL-12 production also proves to be mediated by a MyD88-independent pathway, our data would suggest that apoE possibly can regulate MyD88-dependent and/or -independent signaling. However, this would require that, in the case of the MyD88-independent pathway, the regulation would be restricted to a subset of target genes, ie, IL-12 but not IL-6 or TNF-α. On the other hand, if TLR3-induced IL-12 production proves to be a MyD88-dependent response, our data would be more consistent with the hypothesis that apoE-mediated cytokine production is occurring predominantly via a MyD88-dependent TLR signaling pathway.

Our observation of exaggerated cytokine production in apoE-deficient mice compared with wild-type mice in response to only certain TLR agonists, in addition to possibly being explained by selective effects on specific signaling pathways, may also be attributable to selective regulation of different cell types capable of producing IL-12. Some of the most important producers of IL-12 include dendritic cells, neutrophils, and macrophages. It is known that the initial IL-12 response to *T. gondii* infection is perpetrated by dendritic cells and/or neutrophils.22–24 The fact that we found no differences in IL-12 production between STAg-stimulated apoE<sup>−/−</sup> and wild-type mice is consistent with the possibility that dendritic cells and neutrophils may not be subjected to

Figure 3. Liver-derived circulating apoE is sufficient to regulate the type I inflammatory response. Effect of reconstitution of human apoE on plasma inflammatory cytokines in apoE knockout mice at peak times of expression: TNF-α at 1 hour (A), IL-6 at 3 hours (B), IL-12p40 at 3 hours (C), and IFN-γ at 6 hours (D). Wild-type AAV-lacZ (cross hatched bars), apoE knockout mice AAV-lacZ (black solid bars), and apoE knockout AAV-hapoE (cross bars). The results represent the mean of 4 to 6 mice per group ±SEM. P<0.05: *comparison between AAVlacZ groups; #comparison between apoE<sup>−/−</sup> lacZ and apoE<sup>−/−</sup> hapoE groups.
regulation by apoE as opposed to macrophages. Further support for this hypothesis is provided by the fact that human TLR3 is only expressed on immature dendritic cells in response to poly I:C stimulation, whereas mouse macrophages express high levels of this receptor that can be further induced by multiple stimuli.\(^{1,21-27}\) These circumstances poise the macrophage to be the primary IL-12 producer in response to early poly I:C stimulation. Coupling these observations with the high levels of apoE expression in these cells and the importance of macrophages in atherosclerosis, it seems likely that the macrophage is the primary cell subjected to suppression of IL-12 production by apoE. We are currently investigating this possibility.

ApoE modulation of IL-12 and IFN-γ production can shape the nature of the inflammatory response, suppressing the synergy between the innate and adaptive immune systems that helps to promote inflammation in the burgeoning atherosclerotic plaque. Characterizing the antiinflammatory nature of apoE and the mechanism by which it acts is critical for continuing to develop novel treatments for atherosclerotic disease. Our results clearly establish a selective role for apoE in the modulation of the type I inflammatory response in vivo and provide a scaffold on which to further investigate the mechanism by which this phenomenon occurs.

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**References**


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