Apolipoprotein E-deficient mice have impaired innate immune responses to *Listeria monocytogenes* in vivo

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Abstract **Apolipoprotein E (apoE) influences both innate and acquired immunity in cultured cells. To determine whether apoE affects the immune system in vivo, *Listeria monocytogenes* (LM) was administered intraperitoneally (10^4 c.f.u.) to congenic C57BL/6 apoE −/− and +/+ mice (n = 12 in each group). Survival was assessed daily for 5 days. Deficiency of apoE significantly increased death by day 5 (P = 0.03). The majority of deaths occurred at day 4. Extent of infection after LM administration was assessed at day 3 by determining colony counts in hepatic and splenic extracts. ApoE+/+ mice had very low colony counts in both spleen and liver [mean ± SE: 2.0 ± 0.5 and 0.7 ± 0.2 ($\times 10^4$), respectively, n = 8 in each group]; while apoE−/− mice had significantly increased counts in both spleen and liver [64 ± 51 and 98 ± 93 ($\times 10^4$), *P* = 0.05 and 0.03]. Serum concentrations of TNF-α were significantly increased in apoE−/− mice at day 3 compared to apoE+/+ mice (127 ± 43 pg/ml versus 20 ± 17, *P* = 0.003). LM induced more hepatic damage in apoE−/− mice compared to apoE+/+ mice as judged by increased serum concentrations of alanine aminotransferase at day 1 (apoE−/− 301 ± 45 U/ml, apoE+/+ 101 ± 9 U/ml, *P* = 0.01). The increased proliferation and mortality from LM in apoE−/− mice occurred prior to the initiation of acquired immune responses. Therefore, apoE-deficient mice have an impaired innate response to infection by LM.—**Roselaar, S. E., and A. Daugherty. **Apolipoprotein E-deficient mice have impaired innate immune responses to *Listeria monocytogenes* in vivo. *J. Lipid Res.* 1998.** 39: **1740–1743.**

Supplementary key words macrophages • immunity

Although the importance of apolipoprotein E (apoE) in the metabolism of plasma lipoproteins is well appreciated, there is increasing evidence that apoE is involved in many other physiological processes (1). Based on studies in cultured cells, apoE may influence both innate and acquired immunity. Effects on innate immunity have been demonstrated by the ability of apoE to inhibit stimulation of cultured neutrophils by urate crystals (2). Effects of apoE on the acquired immune system were originally defined with the demonstration that a specific LDL subfraction, designated LDL-In, inhibited lymphocyte proliferation (3). Subsequent studies demonstrated that apoE was the component of LDL-In that inhibited proliferation (4). Purified apoE also inhibits interleukin-2 and -4-stimulated lymphocyte proliferation (5, 6). Furthermore, mono-, di-, and multimeric forms of apoE peptides, between residues 141 to 155, inhibit lymphocyte proliferation (7, 8). Despite these several studies demonstrating an effect of apoE on the immune system, the relevance of these findings in cultured cells have not been defined in vivo.

The intravenous or intraperitoneal administration of *Listeria monocytogenes* (LM) to mice provokes a well-defined sequence of events that involves cells from both the innate and acquired immune system (9, 10). Administered LM distributes rapidly, primarily to liver and spleen. In liver, initially bacteria are phagocytosed primarily by Kupffer cells. After initial bactericidal activity, surviving intracellular bacteria grow logarithmically. Neutrophils infiltrate the liver during the first 24 h of infection. During the next 3 days, neutrophils are replaced by monocyte-derived macrophages. The priming of macrophages for cytocidal activity is dependent on interferon-γ (IFN-γ) produced by natural killer cells. After macrophage activation there is a decline in bacterial number and the development of sterilizing immunity mediated by both CD4+ and CD8+ T-lymphocytes.

The aim of the present study was to determine whether apoE deficiency modified immune responses to the administration of LM. The response to a bacteria load was defined in apoE−/− mice and compared to apoE+/+ animals of a congenic strain (11, 12). We demonstrate that apoE−/− mice are dramatically less resistant to LM infection than congenic apoE+/+ mice, and show that apoE is an important modulator of innate immunity in these mice.

Abbreviations: LM, *Listeria monocytogenes*; c.f.u., colony forming units; apoE, apolipoprotein E; TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ; NK, natural killer.

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METHODS

Animals

ApoE−/− mice, backcrossed 10 generations into a C57BL/6J background, and wild type apoE+/+ C57BL/6J mice were obtained from Jackson Laboratories (Bar Harbor, ME). Mice were housed in specific pathogen-free rooms, on a 12-h light–dark cycle and fed a normal laboratory diet (Ralston Purina, St. Louis, MO). Animals were 8 to 12 weeks of age at the initiation of these studies. All procedures were approved by the Washington University Animal Studies Committee.

Infection with LM

LM were diluted in 0.9% NaCl from a stock culture containing 1.25×10^8 colony forming units (c.f.u.) and injected intraperitoneally. After 1, 3, or 5 days, surviving mice were anesthetized by metaphane inhalation, bled by retro-orbital puncture, and killed by cervical dislocation. Plasma was separated by centrifugation and stored at −70°C.

Survival of mice after administration of LM

To determine whether the ability of mice to survive infection with LM was related to the presence of apoE, apoE−/− mice (n = 12) and apoE+/+ mice (n = 12) were administered LM 10^4 c.f.u. by intraperitoneal injection and observed twice daily for 5 days. Expired mice were removed from surviving animals.

Spleen and liver *Listeria* colony counts

Seventy-two h after infection, mice were killed by cervical dislocation under metaphane anesthesia, placed supine, and the peritoneal cavity was opened aseptically. A cholecystectomy was performed to remove the high number of bacteria in bile, and the remaining liver and spleen were placed in sterile sodium phosphate buffer on ice (NaCl, 145 mm; Na_2HPO_4, 8.45 mm; NaH_2PO_4, 15.8 mm; Triton-X-100, 0.05%). Organs were disrupted in a Dounce homogenizer, and diluted aliquots were inoculated in triplicate on Brain Heart Infusion agar (Difco Laboratories, Detroit, MI). Inoculated plates were incubated at 37°C in room air overnight and colonies were counted after 24 h.

Histology

ApoE−/− and +/+ mice were infected with LM. Livers and spleens were retrieved 24, 48, or 72 h after intraperitoneal infection. Slices (5 mm) of liver with an area of approximately 2 cm^2 and whole spleens were preserved for histological analysis by fixation in 4% (w/v) paraformaldehyde/PBS and embedded in paraffin. Sections of liver and spleen (4 μm) were stained with hematoxylin and eosin and examined for the presence of neutrophil infiltrates and micro-abscess formation by a blinded observer.

Assay of serum tumor necrosis factor (TNF)-α

TNF-α concentrations were determined by solid-phase sandwich enzyme-linked immunosorbent assays (Catalog no. 3012, Biosource, Camarillo, CA) according to the manufacturer's instructions. Samples from all experiments were stored at −20°C and analyzed in a single assay.

Assay of serum aminotransferase activity

Assays for serum concentrations of alanine aminotransferase were performed by the Core Laboratory for Clinical Studies at Washington University, using photometric assays on an Hitachi 917 autoanalyzer.

Statistics

Data are represented as means ± SE. Numbers of bacterial colonies growing from homogenates of spleen or liver from apoE−/− and +/+ mice and TNF-α concentrations were compared by the Mann-Whitney rank sum test for non-parametric data, using SigmaStat (Jandel Scientific, San Rafael, CA). Differences in survival between −/− and +/+ mice were analyzed using Fisher's two-tailed exact test. Differences in concentrations of serum aminotransferases between −/− and +/+ mice were analyzed by one-way analysis of variance using the Tukey test.

RESULTS

Because the genetic background of mice has a dramatic effect on the response to LM, initial experiments were performed to determine the LD_{50} in C57BL/6 mice, the strain into which the apoE deficiency was backcrossed. C57BL/6 apoE +/+ mice were administered 10^5, 10^6, or 10^7 c.f.u. LM in 1 ml of sterile saline and observed. All mice died 3 days after administration of 10^7 c.f.u. Administration of 10^6 or 10^5 c.f.u. resulted in 60% mortality after 4 and 7 days, respectively. Therefore, for all subsequent experiments, mice were administered 10^4 c.f.u. intraperitoneally.

Intraperitoneal injection of LM (10^4 c.f.u.) had no immediate grossly observable effect on either apoE−/− or +/+ mice. Both groups of mice showed normal activity and feeding for the first 24 h of infection. However, soon after, apoE−/− mice were huddling more and becoming lethargic. By day 3 there was a 42% mortality in apoE−/− mice. In contrast, apoE+/+ mice remained generally healthy and there were no deaths during the first 3 days ($P = 0.03$; **Fig. 1**). There was one death in apoE −/− mice at day 5.

As preliminary experiments showed a direct relationship between the number of LM administered and mortality, we sought to determine whether the difference in mortality between apoE−/− and +/+ mice was associated with differences in proliferation of LM in spleen and liver. These assays were performed prior to any deaths in the groups (72 h) so there would be no "survivor" bias in these data. ApoE+/+ mice had low colony counts in both spleen and liver [2.0 ± 0.5 and 0.7 ± 0.2 (×10^4), respec-

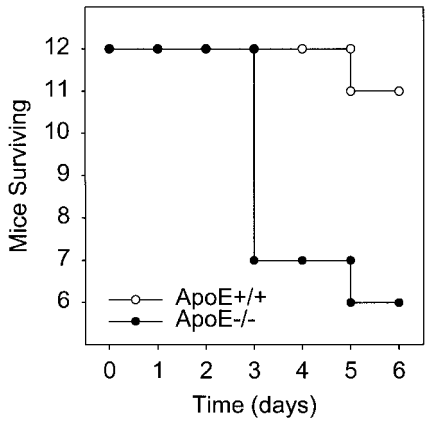

Fig. 1. Kaplan Meyer survival plots of congenic apoE−/− (closed circles) and +/+ (open circles) mice after intraperitoneal infection with 10^4 c.f.u. of LM. Twelve animals in each group were injected and observed twice daily for 6 days. There was a statistically different difference in survival at 5 days, $P = 0.03$.

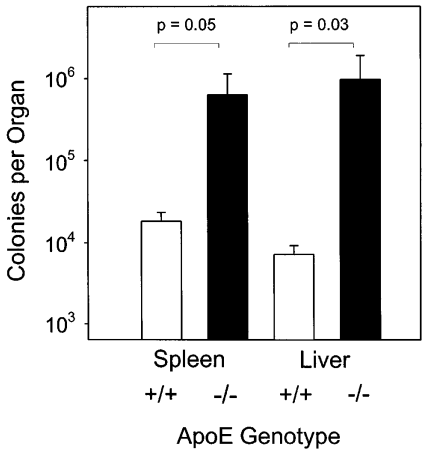

Fig. 2. Colonies of LM cultured from whole livers and spleens of apoE−/− (solid histobars) and +/+ (open histobars) mice 72 h after intraperitoneal administration of 10^4 c.f.u. Both groups contained eight animals. Histobars represent means and error bars represent SE. Differences between groups were statistically significant by a Mann Whitney Rank Sum test.

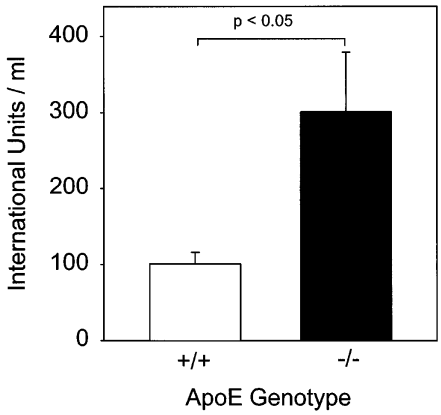

Fig. 4. Serum concentrations of alanine aminotransferase 24 h after intraperitoneal administration of 10^4 c.f.u. LM in apoE−/− (solid histobar) and +/+ (open histobar). Three mice in each group were analyzed. Histobars represent means and error bars represent SE. Statistically significant differences are noted.

tively, n = 8 in each group]; while apoE−/− mice had significantly increased counts in both spleen and liver [64 ± 51 and 98 ± 93 ($\times 10^4$) colonies, $P = 0.05$ and 0.03, respectively] by Mann Whitney Rank Sum test (**Fig. 2**).

TNF-α is produced by activated macrophages and the degree of macrophage activation in response to infection was examined by determining TNF-α concentrations in serum 72 h after infection. After 72 h, at which point all mice in each group survived, apoE−/− and +/+ mice had concentrations of TNF-α that were (mean ± SEM) 127 ± 43 pg/ml and 19 ± 17, respectively (n = 8 in each group, $P = 0.003$ Mann Whitney Rank Sum test) (**Fig. 3**).

Alanine aminotransferase enzyme concentrations were measured in serum as a biochemical assessment of early hepatic inflammation. LM infections in apoE-deficient mice were associated with a dramatic increase in enzyme concentrations in serum (**Fig. 4**).

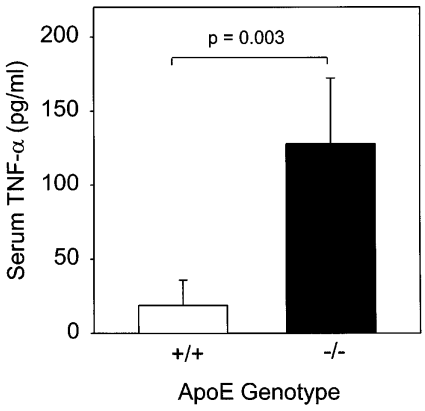

Fig. 3. TNF-α concentration in serum of apoE−/− (solid histobar) and +/+ (open histobar) mice, 72 h after intraperitoneal administration of 10^4 c.f.u. LM. Histobars represent means and bars represent SE. Serum samples from eight mice in each group were analyzed. Statistically significant differences are noted.

Hematoxylin- and eosin-stained sections of livers from apoE+/+ and −/− mice infected with 10^4 c.f.u. LM for 24, 48, and 72 h were examined for the presence of neutrophil infiltrates and micro-abscess formation. Between one and three hepatic micro-abscesses were visible per section in all groups, with no qualitative or quantitative difference discernable in the architecture or number of abscesses (data not shown).

DISCUSSION

Infection of mice with LM is a well-characterized model of innate and acquired immune responses. The initial response of immuno-competent mice to infection with LM is mediated by neutrophils. Macrophages are activated 48 h after infection by a process that is dependent upon secretion of IFN-γ by NK cells (13). Activated T lymphocytes, constituting the acquired immune response to infection, are detectable after 3 days. The major finding of this work is that apoE−/− mice failed to suppress proliferation of LM in the early stages of infection which results in premature death. As the immune response to LM in the first 3 days after infection is mediated by neutrophils and NK cells which enhance the macrophage response, these findings indicate that apoE deficiency is associated with an inadequate innate immune response.

Serum concentrations of TNF-α were significantly increased in apoE−/− mice compared to wild type mice which is consistent with enhanced macrophage activation 3 days after infection. This result would appear to be paradoxical as enhanced macrophage activation would be assumed to augment the extent of bacterial killing. The reason for this disparity is unknown, but may reflect an activation of phagocytes that have an attenuated capacity to eliminate the LM. Others have demonstrated that macrophage activation after LM infection is dependent upon IFN-γ produced by NK cells and therefore increased serum TNF-α concentrations are consistent with enhanced IFN-γ release

by NK cells in apoE−/− mice (14, 15). Although macrophage activation is an important component of the response to LM, it is insufficient for clearance of the organism as reflected by the increased mortality of apoE−/− mice.

Despite observed differences in serum concentrations of both TNF-α and hepatic enzymes, there was no difference in the numbers or architecture of hepatic micro-abscesses. Thus, we conclude that apoE−/− mice are able to form inflammatory micro-abscesses, but their ability to contain LM proliferation is impaired, resulting in overwhelming infection and death. It remains possible that progressively increasing doses of LM would reveal a difference in micro-abscess formation between apoE+/+ and −/− mice, at a point where the physiological differences in responses reported here were manifested histologically.

ApoE −/− mice have a pronounced hyperlipidemia even when maintained on a normal laboratory diet (12, 16). Most of this excessive plasma cholesterol is transported in very low density lipoproteins. The contribution of the hyperlipidemia in itself to the changes in innate responses is not currently known. Unfortunately, it is difficult to separate the deficiency of apoE per se on changes in innate immune responses versus secondary effects that may be attributable to the endogenous hyperlipidemia. Other mice can be rendered hypercholesterolemic, for example, in low density lipoprotein (LDL) receptor −/− mice fed fat and cholesterol-enriched diets (17). However, as this model has elevation predominantly in LDL, this would not necessarily permit comparisons to apoE −/− mice.

This investigation was initiated because of data from cell culture studies indicating that apoE suppressed proliferation of lymphocytes, a cell type that is thought to be involved in many stages of the atherogenic process. Definition of a role for apoE in lymphocyte characteristics would be critical to interpreting the recent reports of immune deficiencies on the development of atherosclerosis (18–20). However, because of the time course of the response to LM infections, we were only able to determine a deficiency of innate immunity in these mice. Other strategies will be needed to define the status of the T lymphocyte response in apoE-deficient mice.

In summary, using a well-characterized model of innate and acquired immunity, these data show that apoE is essential for normal innate immune function in vivo. It remains to be determined which cells are affected in vivo by apoE, and how such effects may impact the development of atherosclerosis.

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