Apolipoprotein E knock-out mice are highly susceptible to endotoxemia and *Klebsiella pneumoniae* infection

Natasja de Bont,* Mihai G. Netea,* Pierre N. M. Demacker,* Ineke Verschueren,* Bart Jan Kullberg,* Ko Willems van Dijk,† Jos W. M. van der Meer,* and Anton F. H. Stalenhoef[1,*]

Division of General Internal Medicine,* Department of Medicine, University Hospital Nijmegen, 6500 HB Nijmegen, The Netherlands, and Department of Human Genetics,† Leiden University Medical Centre, Leiden, The Netherlands

Abstract Lipoproteins are able to neutralize bacterial lipopolysaccharide (LPS) and thereby inhibit the proinflammatory cytokine response. In a previous study, we demonstrated that hypercholesterolemic low density lipoprotein receptor knock-out (LDLr−/−) mice are protected against lethal endotoxemia and gram-negative infection. In the present study we investigated the susceptibility of apolipoprotein E knock-out mice (apoE−/−) to LPS and to *Klebsiella pneumoniae*. These mice have increased plasma lipoprotein concentrations in the very low density lipoprotein (VLDL)-sized fraction. Despite 8-fold higher plasma cholesterol levels compared to controls, and in contrast to LDLr−/− mice, apoE−/− mice were significantly more susceptible to endotoxemia and to *K. pneumoniae* infection. Circulating TNFα concentrations after intravenously injected LPS were 4- to 5-fold higher in apoE−/− mice, whereas IL-1α, IL-1β, and IL-6 did not differ. This TNF response was not due to an increased cytokine production capacity of cells from apoE−/− mice, as ex vivo cytokine production in response to LPS did not differ between apoE−/− and control mice. The LPS-neutralizing capacity of apoE−/− plasma was significantly less than that of controls. Most likely, the absence of apoE itself in the knock-out mice explains the failure to neutralize LPS, despite the very high cholesterol concentrations.—de Bont, N., M. G. Netea, P. N. M. Demacker, I. Verschueren, B. J. Kullberg, K. W. van Dijk, J. W. M. van der Meer, and A. F. H. Stalenhoef. **Apolipoprotein E knockout-mice are highly susceptible to endotoxemia and *Klebsiella pneumoniae* infection. *J. Lipid Res.* 1999.** 40: **680–685.**

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Endotoxin, the lipopolysaccharide (LPS) component of the outer membrane of gram-negative bacteria, is the major pathogenic factor in gram-negative sepsis (1). When infused in vivo, LPS induces hypotension, disseminated intravascular coagulation, and renal, hepatic and cerebral damage, which may lead to shock and death. These actions are mediated by the production and release of proinflammatory cytokines such as tumor necrosis factor α (TNFα) and interleukin-1β (IL-1β) by monocytes and macrophages.

LPS forms complexes with all major lipoprotein classes in plasma, including chylomicrons, very low density lipoproteins (VLDL), low density lipoproteins (LDL), high density lipoproteins (HDL), and lipoprotein [a] (LP[a]) (2–5). Binding of endotoxin to any of these lipoproteins results in neutralization of LPS and reduced release of TNFα, IL-1β, and IL-6 (4, 6, 7). Recent studies have shown that hyperlipoproteinemia in genetically modified mice (8, 9) as well as infusion of reconstituted HDL (rHDL) in humans (10, 11) results in a diminished cytokine response, leading to protection against the toxic effects of endotoxemia. Other studies have demonstrated that triglyceride-rich lipoproteins, such as chylomicrons and VLDL, also have the capacity to inactivate LPS and prevent endotoxin-induced death in rodents (3, 12–14).

It is not well understood which lipoprotein component is most important for LPS neutralization. The ability of lipoproteins to bind and inactivate LPS is modulated by apolipoproteins. Both apolipoprotein (apo) A-I, present on HDL, and apoE, present on chylomicrons, VLDL, HDL, and IDL, are capable of directly inactivating endotoxin and decreasing LPS-induced cytokine release (14–17). An additional mechanism may be represented by the lipid–lipid interactions between the lipid A component of LPS and cholesterol, triglycerides and/or phospholipids, leading to binding and neutralization of LPS (9, 18).

In a previous study with LDL receptor-deficient (LDLr−/−) mice, we demonstrated that these mice with a hypercholesterolemia due to 7- to 9-fold increase of intermediate density lipoprotein (IDL) and LDL are protected against Gram-negative infections (8). The aim of the present study was to investigate the relative importance of the lipid component of VLDL and apoE for binding and neutralization of LPS. For this purpose, we investigated the

Abbreviations: VLDL, very low density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins; apo E−/−, apolipoprotein E deficient; LDLr−/−, LDL receptor deficient; CFU, colony forming units.

[1] To whom correspondence should be addressed.

susceptibility to endotoxin and *K. pneumoniae* of mice with a gene disruption for apo E. Due to the lack of apoE, these mice have hyperlipidemia as a result of decreased clearance and high levels of VLDL (19). As cholesterol-rich LDL and HDL are able to neutralize LPS (12), one would expect cholesterol-rich VLDL to neutralize LPS as well, rendering these mice resistant to LPS like the hypercholesterolemic LDLr−/− mice (8).

MATERIALS AND METHODS

Animals

Homozygous apolipoprotein E-deficient (apoE−/−) mice (6–8 weeks old) were used (Transgenic Facility of Leiden University Medical Center, Leiden, The Netherlands (19)). For the experiments, weight-matched C57BL/6J mice were selected. The animals were kept under specific pathogen-free conditions. The experiments were approved by the ethics committee on animal experiments of the Catholic University Nijmegen.

Lipid measurements

For all mice, total and lipoprotein specific cholesterol and triglycerides in EDTA plasma were determined enzymatically with a Hitachi 747 analyzer 4 days preceeding the experiments mentioned below. For determination of chemical composition and lipoprotein cholesterol and triglyceride concentrations, samples from five mice were pooled. To isolate the VLDL + IDL fraction, plasma was brought to a density of 1.019 g/ml with d = 1.10 g/ml and centrifuged for 18 h at 36,000 *g*. After aspiration of the upper layer, the d > 1.019 g/ml fraction was brought to a density of 1.070 g/ml with d = 1.225 g/ml to isolate the LDL fraction during 24 h at 36,000 *g*. After aspirating LDL from the top layer, HDL was separated from the serum proteins by centrifugation for 24 h at 39,000 *g* after raising the density to 1.18 g/ml. Subsequently, cholesterol and triglyceride concentrations in all of the lipoprotein fractions were determined with the Hitachi 747 analyzer. Phospholipids and free cholesterol in all fractions were measured using a kit (Boehringer Mannheim, Germany). Finally, protein concentrations were ascertained by the method of Lowry et al. (20) and cholesteryl esters were calculated by subtracting free cholesterol from total cholesterol concentration.

Lethality studies

Lipopolysaccharide (LPS; *Escherichia coli* Serotype 055:B5) was obtained from Sigma Chemical Co. (St. Louis, MO). Groups of at least 10 mice were injected iv into the retro-orbital plexus with LPS (dose: 2 mg in 100 μl PBS/mouse). Survival in both groups was assessed daily for at least 7 days.

LPS-induced cytokine production in vivo

In 3 separate experiments, apoE−/− and control mice were injected iv into the retro-orbital plexus with LPS (50 μg in 100 μl PBS/mouse). After 90 min (for TNFα production) and 4 h (for IL-1α and IL-1β production), groups of at least five animals were bled from the retro-orbital plexus into EDTA-containing tubes. Tubes were centrifuged for 5 min at 13,000 *g* and cytokines were measured in the plasma.

Ex vivo cytokine production

In three separate experiments, resident peritoneal macrophages were isolated from groups of at least five apoE−/− and control mice. Subsequently, secreted and cell-associated production of TNFα, IL-1α, and IL-1β by these cells was determined as described elsewhere (8), upon stimulation with 100 ng/ml LPS for 24 h. Samples were stored at −70°C until assay.

Klebsiella pneumoniae infection *K. pneumoniae*

(ATCC 43816), a strain that produces a lethal infection in normal mice, was injected iv into the retro-orbital plexus (0.5×10^5 CFU/mouse) of apoE−/− and control mice. After 90 min, subgroups of five mice were killed and blood was collected from the retro-orbital plexus for the measurement of cytokine concentrations. In a separate group of mice, survival was assessed twice a day.

Cytokine measurements

TNFα, IL-1α, and IL-1β concentrations were measured by specific radioimmunoassays (RIAs) described previously (21). IL-6 concentrations were determined using a commercial ELISA kit (Biosource, Europe S.A.). Detection limits were 0.02 ng/ml for IL-1α and IL-1β, and 0.04 ng/ml for TNFα. The accuracy of the cytokine assays was determined using reference preparations: murine recombinant TNFα, IL-1α, and IL-1β obtained from the National Institute of Biological Standards and Control (NIBSC, Hertfordshire, UK), for the IL-6 ELISA and, for the RPMI samples, control pools were made at our laboratory. All samples of each experiment were analyzed in the same run in duplicate. Using various batches of tracers the bias of all cytokine assays was <20%; interassay variation ranged between 10 and 13% depending on the concentration (n = 22).

LPS measurements

To determine whether the clearance of LPS in apoE−/− mice is different from the clearance of LPS in C57BL/6 mice, six animals of each group were bled 10 min after LPS injection and five animals of each group after 90 min and 4 h into LPS-free EDTA-containing tubes. Tubes were centrifuged for 5 min at 13,000 *g* and plasma was transferred to clean LPS-free tubes. Subsequently, a chromogenic LAL assay (Endosafe Inc., Charleston, SC) was used to measure endotoxin concentrations. Assays were performed according to the manufacturer's instructions.

LPS neutralizing capacity

In order to assess the capacity of plasma of apoE−/− and C57BL/6 mice to neutralize exogenous LPS in vitro, blood of apoE−/− mice and their controls was drawn by cardiac puncture and collected in LPS-free EDTA-containing tubes (Beckton Dickinson). Tubes were centrifuged for 10 min at 1800 *g*. Subsequently, plasma was centrifuged in an Eppendorf centrifuge at 13,000 *g* to acquire platelet-free plasma. LPS (100 ng/ml) was preincubated with the plasma at 37°C. After 24 h, human peripheral blood mononuclear cells (obtained by Ficoll-separation) were added to the LPS and incubated at 37°C once again. After an additional 24 h incubation, cytokines in the supernatant were measured by RIA and the production was expressed per ml of medium containing 10^6 cells.

Statistical analysis

Survival data were analyzed using the Kaplan-Meyer log rank test. Differences in concentrations of cytokines were analyzed using the Mann-Whitney U test. Differences in lipid concentrations were analyzed with the Students' *t*-test. Differences were considered significant at $P < 0.05$.

RESULTS

Concentrations of lipids, lipoproteins, and relative chemical composition

Total plasma cholesterol concentrations were more than 8 times higher in apoE−/− mice than in control C57BL/6

TABLE 1. Plasma and lipoprotein cholesterol and triglyceride concentrations (mmol/l) in apoE−/− and control mice

	Cholesterol		Triglycerides	
	ApoE−/−	Controls	ApoE−/−	Controls
	mmol/l		*mmol/l*	
Plasma	16.14 ± 3.73	1.87 ± 0.15[a]	1.13 ± 0.40	0.85 ± 0.30[b]
VLDL	10.2 ± 1.14	0.12 ± 0.03[a]	0.81 ± 0.14	0.48 ± 0.16[c]
LDL	0.60 ± 0.06	0.23 ± 0.03[a]	0.03 ± 0.01	0.04 ± 0.01
HDL	0.47 ± 0.10	1.04 ± 0.10[a]	0.03 ± 0.01	0.03 ± 0.01

Results are given as mean ± SD for 30 animals per group.
[a] $P < 0.0001$, [b] $P < 0.001$, [c] $P < 0.01$ (for comparison between C57BL/6 and apo E−/−).

mice, due to extremely high cholesterol concentrations of the VLDL + IDL fraction and, to a lesser extent, to LDL cholesterol concentrations, whereas HDL concentration was lower (**Table 1**). Total triglyceride concentrations were slightly, though significantly, higher in the apoE−/− mice (Table 1). The higher triglyceride concentrations were reflected in the triglyceride concentrations of the VLDL + IDL fraction (Table 1).

The chemical composition of all lipoprotein fractions showed differences (**Fig. 1**), especially in the cholesterol and triglyceride content of the VLDL + IDL and the LDL fraction. In the apoE−/− mice, cholesterol represented the most abundant component of lipoproteins, whereas control mice have more triglycerides in these fractions (Fig. 1).

Lethality and cytokines in vivo

Survival after an endotoxin injection is depicted in **Fig. 2**. The mortality in the apoE−/− mice (86%) was significantly higher than in controls (41%). Plasma concentrations of TNFα 90 min after LPS administration were more than 4-fold higher in apoE−/− mice than in control animals (**Fig. 3**). The circulating concentrations of IL-1α, IL-1β, and IL-6 were 10- to 100-fold lower than those of TNF, and no differences were detected 4 h after LPS injection (Fig. 3).

Klebsiella pneumoniae infection

After an intravenous injection of 0.5×10^5 CFU of *K. pneumoniae*, apoE−/− mice showed an increased mortality compared to control mice. All apoE−/− mice died, whereas only 40% of the controls were killed by the infection (**Fig. 4**). Ninety minutes after a *K. pneumoniae* inoculum of 5×10^6 CFU TNF concentration in the circulation of apoE−/− mice was higher than those of control mice (12.3 ± 3.4 ng/ml vs. 7.6 ± 3.8 ng/ml), although these differences did not reach statistical significance ($P = 0.056$).

Ex vivo cytokine production

To investigate whether the increased in vivo production of TNFα was due to an increased capacity of peritoneal macrophages to produce cytokines, cells of both mouse strains were stimulated with LPS. No differences were found in either secreted or cell-associated TNFα, IL-1α, or IL-1β between both strains (**Table 2**).

LPS clearance

To test whether the LPS clearance differed in the two mouse strains, we assessed LPS concentrations in the circulation. Ten min after an iv injection of 50 μg LPS, apoE−/− mice showed levels of endotoxin in plasma similar to control mice in the two mouse strains (3.69 ± 0.80 vs. 3.75 ± 1.21 μg/ml). After 90 min, apoE−/− mice showed lower, albeit not significant, levels of endotoxin in plasma compared with control animals (0.51 ± 0.03 vs. 0.66 ± 0.21 μg/ml). Again no differences were seen 4 h after LPS injection (0.34 ± 0.03 vs. 0.37 ± 0.09 μg/ml).

LPS neutralizing capacity

To investigate whether the lipoproteins in the plasma of apoE−/− and control mice were equally potent in binding and neutralization of LPS, preincubation of LPS with plasma from the two mouse strains was performed. After 24 h, human PBMC were added and cytokine production measured after an additional 24 h incubation. IL-1β and TNFα production by PBMC were higher after preincubation of LPS with plasma obtained from apoE−/− mice than with control plasma (**Table 3**), compatible with decreased LPS-neutralizing capacity of lipoproteins from apoE−/− mice.

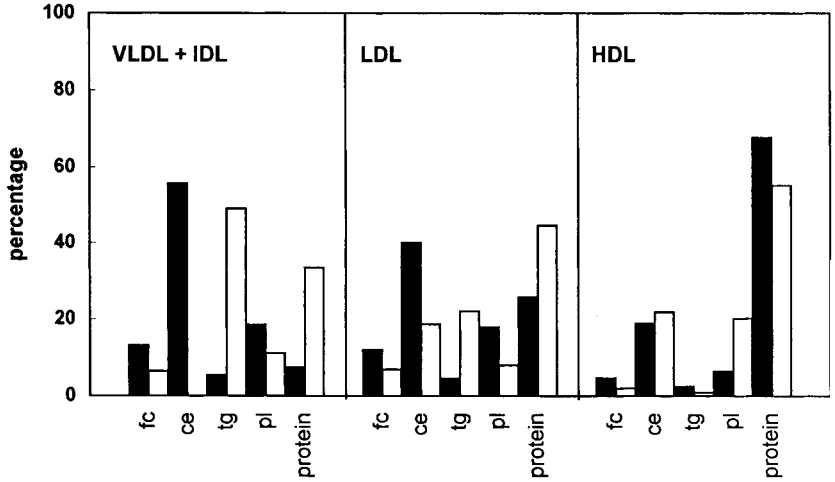

Fig. 1. Chemical composition of lipoprotein fractions (VLDL + IDL, LDL and HDL) of apoE−/− (solid bars) and control mice (open bars). The figure represents the percentage of free cholesterol (fc), cholesteryl esters (ce), triglycerides (tg), phospholipids (pl), and protein of each fraction.

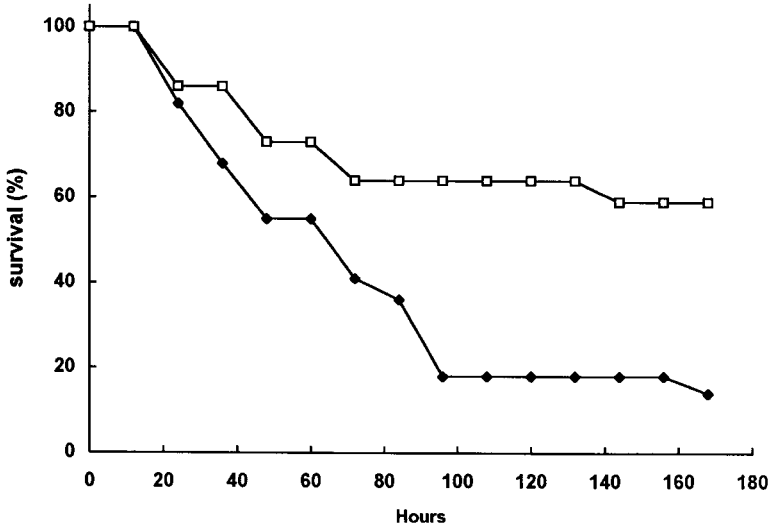

Fig. 2. Survival of apoE−/− (◆) and control mice (□) after LPS (2 mg/mouse iv). Survival was significantly impaired in the apoE−/− mice. The figure shows pooled data of two experiments with at least 10 mice per group each; $P < 0.01$.

DISCUSSION

In the present study we show that hyperlipidemic mice deficient in apolipoprotein E are more susceptible to endotoxemia and to *Klebsiella pneumoniae* infection than control mice. In the apoE−/− mice, the severe cytokinemia, in particular TNFα, is most probably responsible for death. As macrophages of apoE−/− and control mice produced similar amounts of TNFα, the differences do not seem to be due to differences in intrinsic cytokine production capacity.

These results are in accordance with those of Roselaar and Daugherty (22) who demonstrated that apolipoprotein E-deficient mice are more susceptible to *Listeria monocytogenes*. However, their results are in marked contrast to those we obtained in hyperlipidemic LDL receptor knock-out (LDLr−/−) mice that had an increased survival towards a Gram-negative challenge and a dampered proinflammatory endotoxin response by virtue of the endotoxin-neutralizing effects of lipoproteins (8). Moreover, the plasma of the apoE−/− mice appeared to have a low LPS-neutralizing capacity, which was even less than that of normolipidemic control mice. How should these differences be explained?

Basically, two mechanisms may be responsible for neutralization of endotoxin: the increased lipid concentration on the one hand, and the apolipoproteins on the other. Both apoE−/− and LDLr−/− mice have increased plasma lipid concentrations due to either increased cholesterol-rich VLDL in apoE−/− mice or high IDL and LDL in LDLr−/− mice (23). Because both strains have similar elevations in lipoprotein concentrations, and all lipoprotein subfractions have been shown to neutralize LPS (2–7), the discrepancy in LPS neutralization cannot easily be explained by quantitative differences in hyperlipidemia.

The apoE−/− plasma apparently exerts an impaired LPS-neutralizing capacity despite high cholesterol. This is in accordance with results of Harris et al. (12) showing that cholesterol is not necessary for the interaction between lipoproteins and LPS, as a cholesterol-free lipid emulsion, Soyacal® also protected against endotoxin-induced death. On the other hand, infusion of a commercially produced triglyceride-rich particle, Intralipid®, in humans did not result in a reduced cytokine release (24).

It should be taken into account that the lipoproteins in the VLDL-sized fraction of apoE−/− mice have a different chemical composition when compared to controls. Conformational changes may have taken place, resulting

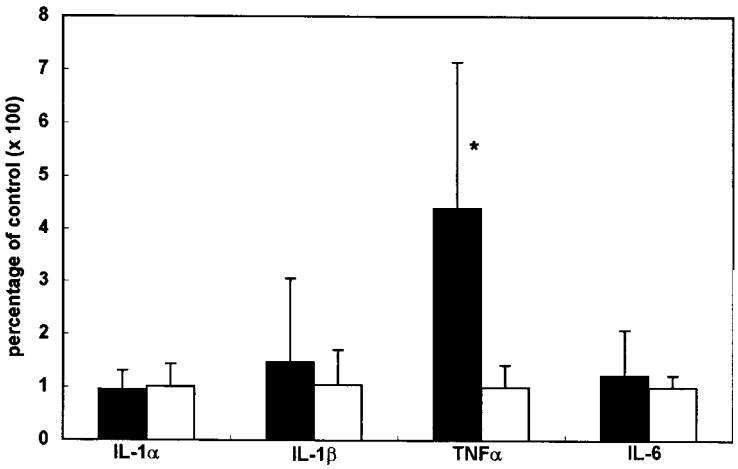

Fig. 3. Plasma cytokine concentrations in apoE−/− (black columns) and control mice (white columns) after 50 μg of LPS iv. The plasma concentrations of TNFα (90 min after the LPS challenge) was significantly higher in apoE−/− mice compared with control mice. No differences in IL 1α, IL-1β, or IL-6 plasma concentrations (4 h after LPS challenge) were detected. The figure shows pooled data of three experiments with groups of at least 5 animals. Data are represented as proportion of the mean cytokine production of control animals; *$P < 0.0001$.

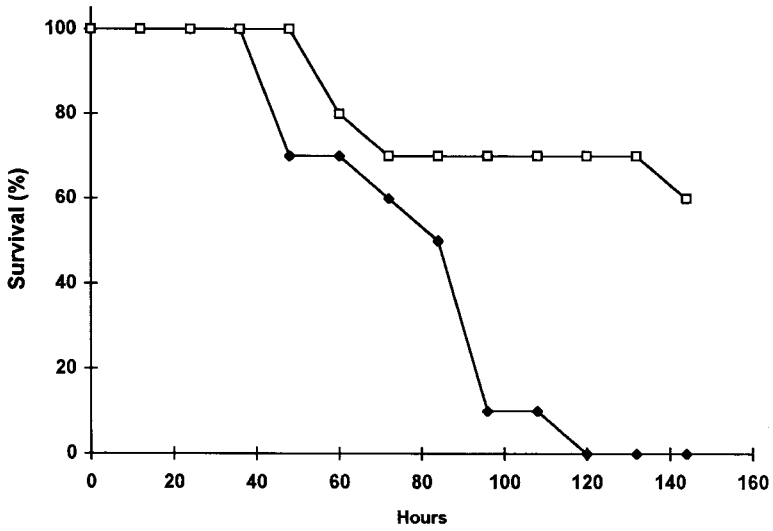

Fig. 4. Survival of apoE−/− and control mice after iv infection with *Klebsiella pneumoniae*. An iv injection of 0.5×10^5 CFU *K. pneumoniae* was given to apoE−/− (◆) and control mice (□). Survival was significantly impaired in apoE−/− mice. The figure shows data of one experiment with 10 mice per group ($P < 0.01$).

in a diminished availability of LPS-binding sites and a decreased neutralizing capacity.

The second possible mechanism concerns the apolipoproteins. Both free apoA-I and apoE have strong LPS neutralizing effects (14–17). In apoE−/− mice, the VLDL + IDL fraction is relatively poor in apolipoproteins. In addition, the excess of lipids carried by the lipoprotein particles in these mice may result in a change of the LPS-binding sites on their apolipoproteins. This, together with a 2-fold decrease in HDL, may be an explanation why LPS in apoE−/− mice is neutralized to a limited extent. In addition to the LPS-binding capacity of apoproteins, apoE seems to be able to neutralize LPS differently. The LPS-detoxifying effects of chylomicrons are thought to be mediated by apoE present on this lipoprotein (14). Free endotoxin, injected iv, is cleared from the plasma by the liver, where it is predominantly taken up by the Kupffer cells resulting in production of cytokines (25, 26). However, when bound to apoE (free or associated with chylomicrons), LPS can be shunted away from these Kupffer cells and directed to the parenchymal liver cells (14), resulting in a reduction of peak cytokine serum levels (17). These observations are in accordance with ours, showing that in apoE−/− mice TNFα plasma concentrations were 4- to 5-fold higher than in controls after LPS challenge. Moreover, the fact that no difference was found in LPS clearance from the plasma between the two mouse strains indicates that the outcome after an LPS injection is determined in the liver and not in the blood. So, most probably, the LPS in apoE deficiency is only directed towards cytokine-producing Kupffer cells, whereas in the control mice a part is directed towards parenchymal cells. How should we explain that we did not find differences in circulating IL-1α, IL-1β, or IL-6 4 h after LPS injection? One explanation could be that TNF and IL-1 production are differentially regulated (27). We have recently found that different LPS receptors seem to be responsible for the production of the various cytokines: CD14-dependent mechanisms mediate TNFα production, while IL-1 synthesis is induced by both CD14-dependent and CD14-independent mechanisms (28). Secondly, IL-1α and IL-1β are less prominent as circulating cytokines than TNFα.

Let us now return to the question how the discrepancy in LPS neutralization between the two hyperlipidemic mouse strains should be explained. In comparison with C57BL/6J mice, LDLr−/− mice have extremely high IDL and LDL concentrations and a 2-fold increased HDL-cholesterol as well (data not shown). ApoE is present on both IDL and HDL, hence increased in LDLr−/− mice. Therefore, absence of apoE in apoE−/− but not in LDLr−/− mice may indeed explain the difference in outcome between the two mouse strains.

TABLE 2. Secreted and cell-associated cytokine production after in vitro stimulation of peritoneal macrophages of apoE−/− and C57BL/6 mice

	Secreted		Cell-associated	
	C57BL/6	ApoE−/−	C57BL/6	ApoE−/−
	ng/ml		*ng/ml*	
IL-1α	0.13 ± 0.03	0.10 ± 0.01	3.62 ± 1.29	2.90 ± 1.29
IL-1β	0.07 ± 0.02	0.05 ± 0.01	0.29 ± 0.07	0.30 ± 0.11
TNFα	0.79 ± 0.19	0.73 ± 0.20	0.19 ± 0.04	0.24 ± 0.08

Peritoneal macrophages (10^5/well) were stimulated with LPS (100 ng/ml) for 24 h at 37°C. The data represent mean ± SD for six animals. The results do not differ significantly for the three cytokines measured.

TABLE 3. Effect of apoE−/− and control plasma on LPS-induced cytokine production

	TNFα		IL-1β	
	ApoE−/−	Control	ApoE−/−	Control
	ng/ml		*ng/ml*	
1	0.59	0.38	2.48	1.31
2	0.27	0.22	1.81	0.65
3	0.44	0.36	1.88	0.84

LPS (100 ng/ml) preincubated with either plasma of apoE−/− or of control mice was added to 5×10^5 PBMC/well and incubated for 24 h at 37°C (n = 3).

In conclusion, in this study we demonstrated that hypercholesterolemia itself is not sufficient for protection against endotoxemia. More likely, the presence of apoE is essential in the process of LPS detoxification, either by catalyzing the binding of LPS to the lipoprotein particle or by directing the LPS to the parenchymal cells away from cytokine-producing Kupffer cells or by both mechanisms.

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