Low-Density Lipoprotein Receptor-deficient Mice Are Protected Against Lethal Endotoxemia and Severe Gram-negative Infections

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Abstract

Lipoproteins can bind lipopolysaccharide (LPS) and decrease the LPS-stimulated production of pro-inflammatory cytokines. We investigated the effect of increased plasma concentrations of low-density-lipoproteins (LDL) on survival and cytokine production after a lethal challenge with either LPS or live Gram-negative bacteria in LDL receptor deficient mice (LDLR—/—). The LDLR—/— mice challenged with LPS had an eightfold increased LD50 when compared with the wild type controls (C57Bl/6j), while tumor necrosis factor α (TNFα) and interleukin-1 α (IL-1α) plasma concentrations were decreased twofold. LDLR—/— mice had significantly lower and delayed mortality than control mice after infection with Klebsiella pneumoniae. No differences in the outgrowth of bacteria in the organs were present between the two groups, while circulating cytokine concentrations were decreased twofold in LDLR—/— mice. In contrast, the LPS-stimulated in vitro production of cytokines by peritoneal macrophages of LDLR—/— mice was significantly increased compared with controls. This increase was associated with enhanced specific binding of LPS to the macrophages of LDLR—/— mice. In conclusion, endogenous LDL can protect against the lethal effects of endotoxin and Gram-negative infection. At least part of this protection is achieved through decreased in vivo production of pro-inflammatory cytokines, in spite of increased cytokine production capacity. (J. Clin. Invest. 1996. 97:1366–1372). Key words: lipopolysaccharide • gram-negative infection • tumor necrosis factor • interleukin-1 • low-density lipoproteins.

Introduction

Mammals infected with Gram-negative bacteria may develop a severe syndrome characterized by hypotension, disseminated intravascular coagulation, and renal, hepatic and cerebral damage. Most of these deleterious effects can be mimicked by infusion of endotoxin, a lipopolysaccharide (LPS) component of the cell wall of Gram-negative bacteria (1). Binding of LPS to various receptors on leukocytes triggers the production and release of proinflammatory cytokines as tumor necrosis factor α (TNFα), interleukin-1α (IL-1α) and IL-1β. In experimental models, treatment of animals with anti-TNF antibodies attenuates the lethal effects of endotoxin or Gram-negative bacteria (2, 3). A protective effect can also be obtained by treatment with either of the natural antagonists of TNF or IL-1, soluble TNF receptors or IL-1 receptor antagonist (4, 5). Thus, these cytokines are thought to play a central role in the pathophysiology of sepsis.

Once LPS enters the circulation, initial interactions with LPS-binding protein (LBP) and lipoproteins play a critical role in subsequent stimulation of pro-inflammatory cytokine synthesis. The LPS-LBP complex is bound by CD14, the cellular receptor for LBP, and this binding stimulates macrophages to release pro-inflammatory cytokines (6, 7). Recent studies also suggest that LBP may facilitate the binding of LPS not only to CD14, but also to lipoproteins (8), either by direct action of LBP or by a two-step reaction in which LPS is first transferred to sCD14 and subsequently to HDL (9). In plasma, LPS binds to the various types of lipoproteins: high-density lipoproteins (HDL) (10), low-density lipoproteins (LDL) (11), very-low-density lipoproteins (VLDL) (12) and chylomicrons (13). Formation of LPS-lipoproteins complexes greatly reduces the binding of LPS to macrophages (14, 15). In vitro, this results in decreased production and release of the pro-inflammatory cytokines TNF, IL-1 and IL-6 (16, 17), and a subsequent loss of LPS toxicity.

The potential protective effect of lipoproteins against LPS-toxicity in vivo has been investigated in recent studies. Preincubation of LPS with lipoproteins prior to injection into animals decreased the mortality (10), while the in vivo infusion of lipoproteins markedly reduced the cytokinemia and mortality after LPS administration (18). Transgenic mice that express human apolipoprotein A-1 at high levels and thus have elevated plasma HDL concentrations are protected against LPS challenge (19). In contrast, hypolipidemic mice are more sensitive to the toxic effects of endotoxin (20).

All these studies have shown a protection by lipoproteins against LPS effects, while only one recent study has reported that hyperlipoproteinemia may beneficially influence severe infections with live microorganisms (21). In a rat model of cecal ligation and puncture, infusion of exogenous chylomicrons reduced both circulating LPS and TNF concentrations and mortality. However, no data regarding the effect of endogenously elevated lipoprotein concentrations on severe infections have been reported. In the present study, we assessed the effects of a lethal LPS Challenge and a lethal Gram-negative infection with Klebsiella pneumoniae in the recently developed...
low density lipoprotein receptor knockout mice (LDLR−/−). These LDLR−/− mice have a markedly increased plasma cholesterol concentration compared with the wild-type controls, due to a 7- to 9-fold increase in intermediate density lipoproteins (IDL) and LDL (22). To study the mechanisms involved in the lipoprotein-induced protection we assessed the bacterial outgrowth in the organs and the cytokine response during infection. In addition, we studied the cytokine production capacity by the peritoneal macrophages of the LDLR−/− and control mice.

Methods

Animals. Homozygous C57BL/6J mice lacking low density lipoprotein receptors (LDLR−/−) and wild-type littermates were obtained from the Jackson Laboratory (Bar Harbor, ME) as mating pairs, and bred in our local facility. For the experiments, 6- to 8-weeks old mice, weighing 20-25 grams, were used. The animals were fed standard laboratory chow (Hope Farms, Woerden, the Netherlands) and housed under specific pathogen-free conditions. The experiments were approved by the ethical committee on animal experiments of the Catholic University Nijmegen.

Lethal endotoxemia model. Lipo polysaccharide (LPS; Escherichia coli serotype O55:B5) was obtained from Sigma Chemical Co. (St. Louis, MO). Groups of LDLR−/− and C57BL/6J mice were injected i.v. in the retroorbital plexus (after anesthetizing with ether) with various amounts of LPS (0.25-2.5 mg/mouse). 90 min after challenge with LPS, five animals from each group were anesthetized with ether and bled from the retroorbital plexus for measurement of TNFα circulating concentrations. Another five animals per group were bled 4 h after LPS for measurement of IL-1α and IL-1β circulating concentrations. In the remaining mice (at least 5 animals/group), survival was assessed daily for 7 d.

Lethal cytokinemia model. To investigate the role of TNF and IL-1 in the LPS-induced mortality, LDLR−/− and control mice received a single i.v. injection of recombinant human TNFα (50 μg/mouse) (kindly provided by Dr. G. Adolf, Ernst-Boehringer Institut, Vienna, Austria), recombinant human IL-1α (10 μg/mouse) (kindly provided by Dr. P. Lomedico, Hoffmann-LaRoche, Nutley, NJ), or a combination of both. Several combinations of recombinant cytokines were used in different experiments: 10 μg TNFα with 1 μg IL-1α, 20 μg TNFα with 5 μg IL-1α, and 50 μg TNFα with 20 μg IL-1α. Survival was assessed every 12 h for 2 d.

Klebsiella pneumoniae infection. Klebsiella pneumoniae (ATCC 43816), a strain that produces a lethal infection in normal mice, was injected i.v. in the retroorbital plexus (10⁶-10⁷ CFU/mouse) into LDLR−/− and control mice (LD50 = 8 × 10⁵ CFU/C57BL/6J mouse). To assess whether the same protection is obtained in a more sustained model of infection, K. pneumoniae 10⁶-10⁷ CFU/mouse was injected intramuscularly (i.m.) (LD50 = 5 × 10⁷ CFU/C57BL/6J mouse) into the left thigh muscle of the animals as described (23). Survival was assessed daily for 14 d, in groups of at least 18 (i.v. infection) or 9 animals (i.m. infection). The surviving animals were sacrificed on day 28 of infection. After 90 min and 4 h, subgroups of five mice were killed and blood was collected from retroorbital plexus for the measurement of cytokine concentrations. The outgrowth of the microorganisms from the blood, liver, spleen and kidneys of the animals was quantified 24 h after the injection of K. pneumoniae. The organs were removed aseptically, weighed, and homogenized in sterile saline in a tissue grinder. Serial dilutions of the homogenized tissues or blood (0.1 ml) were plated on sheep blood agar, and CFU were counted after overnight incubation at 37°C.

To determine the circulating LPS concentrations, in a separate set of experiments groups of 10 LDLR−/− and control mice were infected i.v. or i.m. with 10⁶ CFU K. pneumoniae as described above. 90 min and 4 h after infection, groups of five animals were anesthetized and bled collected by intracardiac puncture into 5-ml pyrogen-free plastic vials (Falcon; Becton-Dickinson Labware, Lincoln Park, NJ) containing 25 IU of pyrogen-free heparin. The tubes were centrifuged at 200 g for 10 min and plasma was collected and stored at −70°C until assay.

Ex vivo cytokine production. Resident peritoneal macrophages were harvested by rinsing the peritoneal cavity aseptically with cold PBS containing 0.38% (wt/vol) sodium citrate. After centrifugation for 10 min, 1800 rpm, at 4°C, cells were resuspended in RPMI 1640 (Dutch modification; Flow Laboratories, Irvine, UK), containing 1 mM pyruvate, 2 mM L-glutamine and 100 μg/ml gentamicin. 10⁷ cells/well were cultured in 96-well microtiter plates (Costar Corporation, Cambridge, MA) in RPMI medium (final volume 200 μl), with or without LPS at a final concentration of 1 μg/ml. No plasma or serum was added to the culture medium. The supernatants were collected after 24 h of incubation at 37°C, and stored at −70°C until assay. To the remaining macrophages, 200 μl RPMI 1640 was added, and the cells were disrupted by three freeze-thaw cycles to determine the cell-associated cytokine contents. The samples were stored at −70°C until cytokine measurements.

The cytokine production capacity of peritoneal macrophages was also measured after stimulation by heat-killed (100°C, 30 min) Staphylococcus aureus (ATCC 25923). The suspension was washed three times in RPMI (final concentration 2 × 10⁵ CFU/ml). 100 μl suspension containing 10⁵ microorganisms in RPMI were added to 10⁵ peritoneal macrophages in 96-well microtiter plates and the cells were incubated 24 h at 37°C. The samples were obtained and stored at −70°C until cytokine measurements, as described above.

Cytokine measurements. TNFα, IL-1α and IL-1β concentrations were determined using specific radioimmunoassays (RIAs) developed in our laboratory (24). Briefly, standards and samples were diluted in RIA-buffer (per liter: 10 mM NaH2PO4, 2 mM EDTA; 1 ml sodium azide (20% w/v/vol); 2.5-gm bovine serum albumin fraction IV (Sigma Chemical Co., St. Louis, MO); 1 ml Triton X-100; 25 ml aprotinin solution (Trasylol, containing 10,000 inhibitor units per milliliter, Bayer BV, Midrecht, The Netherlands) diluted with deionized water milli-Q, pH 7.4. 100 μl of an appropriate monoclonal specific rabbit antisera against the respective mouse cytokine was added to 100 μl of samples and standards. After vortexing, the tubes were incubated for 24 h at 4°C, followed by addition of 100 μl of the respective 125I-labeled tracer. After 20 h, 750 μl RIA buffer containing 9% (w/vol) polyethylene-glycol 6000, and 3% (vol/vol) goat anti-rabbit IgG was added. The tubes were incubated for 20 min at room temperature and then centrifuged at 1,500 g for 15 min. Supernatants were discarded carefully and quickly drained on absorbent paper. Remaining radioactivity was counted in a gamma-counter. The radioactivity in control tubes (the non-specific binding activity) was subtracted from samples and standards. Detection limits were between 0.02 and 0.04 ng/ml. The mean of duplicate determinations was calculated for each sample. 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, Herfordshire, UK). Using various batches of tracers the bias of all cytokine-assays was < 20%; interassay precision ranged between 10 and 13% depending on the concentration (n = 22).

LPS measurements. Plasma samples were diluted 1/100 and 1/600 with pyrogenfree saline and heated at 75°C for 5 min before the limulus assay to remove the effect of serum inhibitors. LPS was determined in platelet-rich plasma by a chromogenic limulus amebocyte lysate essay (Endosafe Inc., Charleston, SC).

Radioactive labeling of LPS. E. coli O55:B5 LPS was labeled with tritium as described previously (25) with minor modifications. Briefly, LPS (5 mg/ml) was oxidized with sodium periodate (1.5 mg/ml) in 50 mM acetic acid buffer, pH 4.0, for 24 h. The remaining periodate was destroyed with ethylene glycol and the LPS was buffer-exchanged and concentrated in an Amicon concentrator (molecular cut-off 10,000 Da). 10 μCi [3H] sodium borohydride (specific activity 1367.5 Ci/mmol) was added to each 1 ml of LPS solution. After incubation at 37°C for 1 h, the labeled LPS was purified on a Sephadex G-25 column (Pharmacia, Sweden) by elution in 0.1 M sodium acetate, pH 4.0, during 24 h. The remaining material was dialyzed against PBS (pH 7.4) and used in the experiments.
IL-1α (2Ci/mmol) was added to the oxidized LPS (5 mg/ml) in 1 mM Tris-HCl, pH 8.0. After a 2 h incubation at room temperature, the reaction was terminated by the addition of 25 mg nonradioactive sodium borohydride. The tritiated LPS was purified on a PD-10 column (Pharmacia, Woerden, The Netherlands). Subsequently, the unincorporated \(^3\)H was removed by repeated concentration in an Amicon concentrator. Elution of the \(^3\)H-labeled LPS preparation on a PD-10 column indicated that more than 95% of the \(^3\)H-label was associated with LPS both in the fresh preparation, as well as in the preparation incubated overnight at room temperature (the temperature of the incubation in the binding studies). The specific activity of this \(^3\)H-LPS was 16,000 cpm/\(\mu\)g.

**Binding of radiolabeled LPS to peritoneal macrophages.** Peritoneal macrophages from LDLR\(^{-/-}\) and C57Bl/6J mice were collected as described above, and \(10^6\) peritoneal macrophages were incubated in 100 \(\mu\)l RPMI 1640 in 0.5 ml Eppendorf plastic cups. No plasma or serum was present in the system. Increasing amounts of \(^3\)H-LPS (10,000–100,000 cpm) were added and the cells were incubated at room temperature for 45 min (26). Cells were spun down (2,000 g, 5 min), washed twice in 100 \(\mu\)l RPMI, solubilized in scintillation fluid and counted in an \(\beta\)-counter. Nonspecific binding was determined in the presence of a large excess of nonradioactive LPS. Specific binding of LPS was defined as the difference between total and nonspecific binding.

**Determination of plasma lipids.** Cholesterol and triglycerides were determined by enzymatic methods on a Hitachi 747 analyzer.

**Statistical analysis.** Survival data were analyzed using the Kaplan-Meyer log rank test (27). Differences in concentrations of cytokines in and organ counts of the microorganisms were analyzed using the Kruskall-Wallis test (28). Differences were considered significant at \(P < 0.05\). All experiments were performed at least twice.

## Results

**Lethal endotoxemia model.** Plasma cholesterol concentrations are presented in Table I. The total cholesterol concentrations were fourfold higher in LDLR\(^{-/-}\) mice at all the time points tested. The plasma triglyceride concentrations differed only marginally in the two mouse strains both 90 min (0.67±0.13 vs. 0.62±0.09 mmol/liter, \(P > 0.05\)) and 4 h (0.50±0.07 vs. 0.42±0.06 mmol/liter, \(P > 0.05\)) after LPS. The survival of the LDLR\(^{-/-}\) mice was significantly increased when compared with C57Bl/6J mice, the estimated endotoxin LD\(_{50}\) dose being 8-times higher (\(P < 0.001\)) (Fig. 1). Mortality occurred in the first 48 h after endotoxin administration, without further mortality during the next days of the follow-up. Plasma concentrations of TNF\(_\alpha\) were significantly lower in LDLR\(^{-/-}\) mice 90 min after LPS administration, than in control animals (1.5±0.5 vs. 3.7±0.9 ng/ml, \(P < 0.02\)) (Fig. 2). Four hours after the endotoxin challenge, IL-1\(\alpha\) concentrations were also lower in LDLR\(^{-/-}\) mice (60±21 vs. 106±26 pg/ml, \(P < 0.05\)), while no differences in IL-1\(\beta\) concentrations were detected (36±9 vs. 37±9 pg/ml, \(P > 0.05\)) (Fig. 2).

**Lethal cytokinemia model.** To investigate whether the protection to a lethal injection of endotoxin is due to decreased susceptibility to proinflammatory cytokines, mice were injected i.v. with pro-inflammatory cytokines. The administration of either 50 \(\mu\)g recombinant human TNF\(_\alpha\) or 10 \(\mu\)g IL-1\(\alpha\) did not induce any mortality. After the administration of a combination of these cytokines, no protection by the high lipoprotein concentrations of LDLR\(^{-/-}\) mice was observed. The mortality was 0% in both mouse strains when a combination of 10 \(\mu\)g of TNF\(_\alpha\) and 1 \(\mu\)g IL-1\(\alpha\) was injected. When a combination of 20 \(\mu\)g TNF\(_\alpha\) with 5 \(\mu\)g IL-1\(\alpha\) was injected the mortality was 20% in LDLR\(^{-/-}\) mice and 33% in the C57Bl/6J mice (\(P > 0.05\)), and when a combination of 50 \(\mu\)g TNF\(_\alpha\) with 10 \(\mu\)g IL-1\(\alpha\) was injected the mortality was 80% in LDLR\(^{-/-}\) mice, and 91% in controls (\(P > 0.05\)). The mortality occurred exclusively during the first 48 h.

**Klebsiella pneumoniae infection.** Cholesterol concentrations during K. pneumoniae infection decreased in both strains of mice. However, they remained significantly higher in LDLR\(^{-/-}\) mice than in control mice (Table I). No significant differences in triglyceride levels 90 min after infection were found between the LDLR\(^{-/-}\) and C57Bl/6J mice (0.79±0.11 vs. 0.86±0.11 mg/dl). Plasma concentrations of both TNF (90 min after an intravenous injection with 1 mg LPS. The plasma concentrations of both TNF (90 min after the LPS challenge) and IL-1\(\alpha\) (4 h after LPS challenge) were significantly decreased in LDLR\(^{-/-}\) mice when compared with control mice. No differences in IL-1\(\beta\) plasma concentrations were detected 4 h after LPS was injected. *\(P < 0.05\); **\(P < 0.02\).
Table I. Plasma Cholesterol Concentrations (mmol/liter) before and after LPS Challenge and K. pneumoniae Infection in LDLR—/— and C57Bl/6J Mice

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<tr>
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<th>LDLR—/—</th>
<th>C57Bl/6J</th>
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<tbody>
<tr>
<td>Before infection</td>
<td>9.55±1.11</td>
<td>2.25±0.45*</td>
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<tr>
<td>After LPS</td>
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<tr>
<td>90 min</td>
<td>5.16±1.53</td>
<td>1.42±0.27*</td>
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<tr>
<td>4 h</td>
<td>4.10±0.69</td>
<td>1.42±0.18*</td>
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<tr>
<td>After K. pneumoniae</td>
<td>5.72±1.05</td>
<td>1.78±0.08*</td>
</tr>
<tr>
<td>4 h</td>
<td>6.27±1.16</td>
<td>1.96±0.17*</td>
</tr>
<tr>
<td>24 h</td>
<td>7.42±0.63</td>
<td>2.54±0.71*</td>
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LDLR—/— and control mice were challenged with LPS (1 mg/mouse) or infected i.v. with 10^6 CFU of K. pneumoniae. Each figure represents mean±SD of five mice. *P < 0.01. ^P < 0.05 (for comparison between C57Bl/6J and LDLR—/—).
In this study we showed that LDL receptor deficient mice were protected against lethal endotoxemia as well as against severe infections with Gram-negative microorganisms, not only after acute i.v. infection, but also in a more subacute model of intramuscular infection with *Klebsiella pneumoniae*. Lipoprotein concentrations in the LDLR−/− mice are genetically elevated, due to increased IDL and LDL (22). Our approach thus differs from other models in which exogenous lipoproteins were administered to obtain hyperlipoproteinemia (10, 11, 18). This intervention was associated with decreased cytokinemia and improved survival during lethal endotoxemia. The same protection was reported in mice in which endogenous lipoproteins were administered to obtain hyperlipoproteinemia (20), who reported increased LPS-induced mortality when serum lipids were decreased by pharmacological agents. However, our study shows that the protection due to increased endogenous LDL can be extended to severe Gram-negative infections with live microorganisms.

In general, mortality to infection is considered to be due to lethal cytokinemia or to functional impairment by excessive growth of microorganisms in the organs of the host. In the model of infection we studied, showing no difference in the number of circulating bacteria or the number of bacteria in the tissues, the protection was probably achieved through the high levels of LDL in the LDLR−/− mice, which are able to neutralize the effects of LPS released by similar bacterial loads as in the control mice, and consequently inhibit the induction of lethal concentrations of cytokines. The crucial role of cytokines in the response to a Gram-negative infection, independently of the bacterial load, has also been documented in our previous studies (29, 30).

The protection against the LPS component of Gram-negative bacteria by lipoproteins is achieved either by direct neutralization of LPS by forming LPS-lipoprotein complexes (8, 9, 10), or by accelerating the clearance of LPS by lipoproteins (11). In this respect, we considered if in our study an increased clearance of LPS or the direct neutralization of LPS by lipoproteins is playing the main role in the protection of LDLR−/− mice during *K. pneumoniae* infection. The LPS concentrations tended to be lower in LDLR−/− mice than in controls when the infection was induced by i.v. injection of *K. pneumoniae*. This may be due to increased LPS clearance, since we determined the LPS concentrations after heating the plasma before the assay, a procedure which liberates lipoprotein-bound LPS (31). However, in the i.m. *K. pneumoniae* infection model, the LPS concentrations were similar in the LDLR−/− and control mice. Since LDLR−/− mice in this model also showed decreased circulating cytokines, as well as decreased mortality.
infection is through direct binding and neutralization by lipoproteins, rather than increased clearance of endotoxin. This is not surprising since the clearance of LDL, which is the main lipoprotein fraction increased in the LDLR−/− mice, is decreased in these mice (22), due to the lack of the receptor.

The increased cytokine concentrations in the circulation during Gram-negative infections are thought to be induced by the LPS component of the cell wall. We have shown that LDLR−/− mice were more LPS tolerant, the LPS LD₅₀ in LDLR−/− mice being increased approximately eightfold. Associated with the increased protection of LDLR−/− mice we observed decreased concentrations of the pro-inflammatory cytokines TNFα and IL-1α. The LDLR−/− mice were not protected against a lethal injection of these cytokines, arguing against a direct inactivation of circulating cytokines by lipoproteins, and against decreased peripheral sensitivity of LDLR−/− mice to TNFα or IL-1. This confirms that the protection of LDLR−/− mice in lethal endotoxemia is due to an inactivation of the bacterial LPS by increased plasma lipoproteins, which in turn results in a blunted response of the pro-inflammatory cytokines.

An interesting observation was made when we evaluated the in-vitro cytokine production capacity of the LDLR−/− mice. We showed that, in contrast to the in-vivo decreased cytokine production after LPS challenge, the in-vitro LPS-stimulated cytokine production capacity of peritoneal macrophages of mice with hypercholesterolemia was higher than in control mice. This increase was observed for all the cytokines tested: TNFα, IL-1α and IL-1β. An explanation for these findings could be up-regulation of LPS receptors on the macrophages of LDLR−/− mice. Indeed, our LPS-binding experiments showed increased specific binding of tritiated LPS to the macrophages of LDLR−/− mice. In normal mice, exposure to small LPS quantities may down-regulate its own receptors on monocytes/macrophages: distinct effects of serum constituents.

An increased cytokine production capacity has also been observed in monocytes of patients with familial hypercholesterolemia (35) and in individuals with high plasma HDL concentrations (36). The results of these studies and our present findings argue for a more general phenomenon of increased cytokine production by monocytes/macrophages of hosts with high circulating lipoprotein concentrations. The clinical importance of this phenomenon remains to be further studied.

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