Circulating Lipoproteins Are a Crucial Component of Host Defense against Invasive Salmonella typhimurium Infection

Mihai G. Netea1,2, Leo A. B. Joosten1,2, Monique Keuter1,2, Frank Wagener3, Anton F. H. Stalenhoef1, Jos W. M. van der Meer1,2, Bart Jan Kullberg1,2*

1 Division of General Internal Medicine, Department of Medicine, Radboud University Nijmegen Medical Center, Nijmegen, The Netherlands; 2 Nijmegen Institute of Infection, Inflammation and Immunity (N4i), Nijmegen, The Netherlands, 3 Department of Pharmacology and Toxicology, Nijmegen Center for Molecular Life Sciences, Radboud University Nijmegen Medical Center, Nijmegen, The Netherlands

Abstract

Background: Circulating lipoproteins improve the outcome of severe Gram-negative infections through neutralizing lipopolysaccharides (LPS), thus inhibiting the release of proinflammatory cytokines.

Methods/Principal Findings: Low density lipoprotein receptor deficient (LDLR−/−) mice, with a 7-fold increase in LDL, are resistant against infection with Salmonella typhimurium (survival 100% vs 5%, p<0.001), and 100 to 1000-fold lower bacterial burden in the organs, compared with LDLR+/+ mice. Protection was not due to differences in cytokine production, phagocytosis, and killing of Salmonella organisms. The differences were caused by the excess of lipoproteins, as hyperlipoproteinemic ApoE−/− mice were also highly resistant to Salmonella infection. Lipoproteins protect against infection by interfering with the binding of Salmonella to host cells, and preventing organ invasion. This leads to an altered biodistribution of the microorganisms during the first hours of infection: after intravenous injection of Salmonella into LDLR+/+ mice, the bacteria invaded the liver and spleen within 30 minutes of infection. In contrast, in LDLR−/− mice, Salmonella remained constrained to the circulation from where they were efficiently cleared, with decreased organ invasion.

Conclusions: Plasma lipoproteins are a potent host defense mechanism against invasive Salmonella infection, by blocking adhesion of Salmonella to the host cells and subsequent tissue invasion.

Introduction

Salmonella infections are a significant cause of morbidity and mortality, despite preventive measures and the availability of antibiotics. A major virulence factor of Salmonella is lipopolysaccharide (LPS) [1,2], and Salmonella strains with a reduced LPS expression have a poor growth under stress conditions and are less virulent [2]. In addition, LPS induces proinflammatory cytokines and is essential for internalization of Salmonella by host cells [3]. Interaction of LPS with cellular receptors is essential and therefore, strategies aimed at blocking this interaction may have a therapeutic potential in invasive infections.

Lipoproteins bind and neutralize bacterial LPS, and they prevent the induction of potentially harmful proinflammatory cytokines such as IL-1β and TNFα [4]. In experimental models, administration of lipoproteins protects against endotoxic shock [5–7]. Low density lipoprotein receptor deficient (LDLR−/−) mice have a 7 times higher LDL-cholesterol level than control mice. We have shown previously that LDLR−/− mice survive longer and have lower proinflammatory cytokine concentrations than control mice after LPS challenge, as well as after infection with Klebsiella pneumoniae [8]. In addition, LDL administration can protect against Gram-negative microorganisms through its neutralizing effects on LPS [9].

Although Salmonella is a Gram-negative organism, the clinical picture and inflammatory response in systemic Salmonella infections (e.g., typhoid fever) differs from that in other Gram-negative sepsis [10]. This is most likely due to the behaviour of Salmonella as facultative intracellular pathogens, and to the fact that the pattern of cytokine induction differs from other Gram-negative infections. In mice, TNFα is undetectable in the circulation until several days after S. typhimurium infection, whereas TNFα rises at 1 hour after extracellular Gram-negative infection [11]. The level of cytokinemia during Salmonella infections does not reach the toxic levels seen in endotoxic shock, and inhibition of TNFα during Salmonella infection worsens the outcome [12,13].

Considering these differences in the pathogenesis of Salmonella and extracellular Gram-negative infections, one would envisage either beneficial effects of lipoproteins on the host resistance to Salmonella through blockade of cellular internalization, or deleterious effects by blocking the induction of cytokines by Salmonella LPS that are required for the activation of host defense. In the
present study, we investigated the effect of lipoproteins on the 
outcome of Salmonella infection.

**Methods**

**Animals**

Homozygous C57Bl/6j mice lacking low density lipoprotein 
receptors (LDLR–/–) and their wild-type littermates (C57Bl/6j 
LDLR+/+) were obtained from Jackson Laboratory (Bar 
Harbour, ME) [8]. Homozygous apolipoprotein E (ApoE)-deficient mice 
with a C57Bl/6j background were obtained from the Transgenic 
Facility of Leiden University Medical Center, Leiden, The 
Netherlands [14]. Six to eight weeks old littermate LDLR+/+ and 
LDLR–/– mice were used, weighing 20–25 grams. The animals 
were fed standard laboratory chow and housed under specific 
pathogen free conditions. The experiments were approved 
by the Ethics Committee for animal experiments at the Radboud 
University Nijmegen.

**Salmonella typhimurium infection**

A serum-resistant strain of S. typhimurium (phage type 510) was 
grown by overnight incubation at 37°C in nutrient broth (BHI Oxoid). Mice were injected i.v. or i.p. with 1×10\(^5\) cfu of S. 
*typhimurium*. Survival was assessed daily for 21 days in groups of at 
least 20 animals. On day 1, 3 and 7 after infection, mice were 
killed by cervical dislocation and blood for cytokines or organs for 
outgrowth of the microorganisms were collected. For this purpose, 
the liver and spleen were removed aseptically, and bone marrow 
was flushed from the femur aseptically with 1 ml of sterile saline. 
The number of viable *Salmonella* organisms was determined by 
plating several dilutions on Brilliant Green agar (BGA) plates. The 
results were expressed as log cfu per gram of tissue.

**Distribution of Salmonella cfu**

In a separate experiment, *Salmonella* cfu (10\(^3\)/mouse) were 
 injected i.v. Distribution was determined in blood, liver and spleen 
after 30, 60, 120 and 360 minutes by plating serial dilutions of 
blood and homogenized tissue samples on BGA plates. Groups of 
5 mice were used for each time point.

**Intracellular killing of S. typhimurium by peritoneal phagocytes**

Phagocytosis and intracellular killing of *S. typhimurium* was 
assessed in vitro using peritoneal macrophages and PMN of 
LRiR+/+ and LDLR–/– mice. Exudate peritoneal neutrophils (PMN) 
were harvested 4 hr after i.p. injection of 10\(^5\) cells with phagocytosed 
bacteria were scraped with a plastic paddle and washed with 200 ml 
distilled H\(_2\)O to lyse the phagocytes. The number of viable bacteria was determined by 
plating serial dilutions on BGA plates.

**In vitro cytokine production**

Resident peritoneal macrophages were harvested from perito-
neal cavity, and cells were resuspended in RPMI 1640 containing 
1 mM pyruvate, 2 mM L-glutamine and 100 μg gentamicin per 
ml, and incubated (10\(^5\)/well) in 96-wells microtiter plates (Costar). 
Heat-killed (30 min, 100°C) *S. typhimurium* (10\(^5\) microorganisms in 
100 μl of RPMI) were added to peritoneal macrophages and 
incubated at 37°C in 5% CO\(_2\). After 24 hr, the supernatants 
were collected and stored at –70°C until assayed. To the macrophages 
in the monolayer, 200 μl of RPMI was added and the cells were 
disrupted by three freeze-thaw cycles to determine the cell-
associated cytokine contents.

**Cytokine measurements**

TNF\(\alpha\), IL-1\(\alpha\) and IL-1\(\beta\) concentrations were determined using specific 
radioimmunoassays (RIA), as previously described [8]. To assess 
cytokine mRNA expression, total RNA from spleen cells 
24 hours after infection was isolated as described [15]. The following 
primers were used for the PCR reactions: GAPDH, sense, 5′-
AACCTCCCTCAAGATTGTGACCA–3′, and antisense, 5′-TCC-
ACCACCCTGTGTGCTGTA–3′; TNF\(\alpha\), sense, 5′-TCATTCA-
CATTTCTATGGCCTC–3′, and antisense, 5′-GGAGTGA-
CAAGGTGACAC–3′; IL-1\(\alpha\), sense, 5′-CATGTTCGCACT-
GACCATCT–3′, and antisense, 5′-TCTCACTGAAACCTGAC-
CCGT–3′; IL-1\(\beta\), sense, 5′-TTGAGCGACCCAAAAGATGCTG-
AAGGTGCTATGTCCTA–3′ (Eurogentec, Seraing, Belgium). After checking the reactions to be in the 
log phase, thirty PCR cycles were performed with sets at 92°C for 30 sec., 
55°C for 30 sec., and 72°C for 90 sec., using a Mastercycler 
5330 (Eppendorf). PCR products were run on 2% agars gels stained 
with ethidium bromide. The gels were scanned on a densitometer 
3030 (Eppendorf). PCR products were run on 2% agars gels stained 
with ethidium bromide. The gels were scanned on a densitometer 
(GS-670, Bio-Rad) and analyzed using Molecular Analyist software 
(Bio-Rad). The relative amount of TNF\(\alpha\), IL-1\(\alpha\) and IL-1\(\beta\) mRNA in 
a sample was expressed as a ratio versus the amount of mRNA for 
the housekeeping gene GAPDH.

**Growth of Salmonella in vitro**

To investigate the effect of lipoproteins on microbial growth in 
in vitro, 0.5×10\(^5\) cfu *S. typhimurium* in 0.5 mL BHI were incubated 
with 0.5 mL of plasma obtained from control C57Bl/6j mice, or 
from LDLR–/– mice and ApoE–/– mice. After 2, 7, 12 and 
24 hours, aliquots of 0.1 mL were removed, serial dilutions were 
plated on BGA agar, and cfu were counted after overnight 
icubation at 37°C.

**Effect of lipoproteins on interaction of Salmonella with monocytes and endothelial cells**

To assess the effect of lipoproteins on the production of 
cytokines, *S. typhimurium* LPS (10 ng/mL; Sigma) and heat-killed 
(30 min, 100°C) *S. typhimurium* (10\(^5\) organisms/mL) were preincu-
badated with lipoprotein-depleted plasma (LPDP) or isolated LDL 
[16] at various concentrations for 60 min, before being added to 
the macrophages of LDLR+/+ mice (10\(^7\)/well). The production of 
TNF\(\alpha\) after 24 hr stimulation was measured as described above, 
and expressed as relative TNF production compared to controls in 
LPDP.

To assess the effect of lipoproteins on the attachment of 
*Salmonella* to vascular endothelial cells, *S. typhimurium* were 
resuspended to 6×10\(^5\)/ml in 0.01 mg/ml FITC (Fluka) in 
0.05 M carbonate-bicarbonate buffer (pH 9.5). After incubation 
for 15 min at room temperature in the dark, FITC-labeled 
*Salmonella* cells were washed twice in PBS containing 1% BSA and 
subsequently incubated with isolated 1.1 mmol/L LDL for 
4 hours, or with LPDP as a negative control. The human 
endothelial cell line (HMEC-1) (CDG Atlanta, GA) was cultured in 
MCDBl51 medium supplemented with 10% fetal calf serum,
Figure 1. LDLR+/+ mice are more resistant to S. typhimurium infection. Survival of LDLR−/− and LDLR+/+ C57Bl/6J mice after i.v. injection of 10^7 S. typhimurium. n = 20/group. doi:10.1371/journal.pone.0004237.g001

EGF (10 ng/ml), hydrocortison (1 µg/ml), and glutamine at 37°C and 5% CO₂. 1x10⁶ HMEC-1 cells were trypsinized and incubated for 1 hour with 3x10⁶ FITC-labeled S. typhimurium, which were preincubated with either LDL or LPDP. After incubation, the non-bound Salmonella was thoroughly washed off, after which the cells were fixedated with 2% paraformaldehyde in PBS and analyzed for binding of FITC-labeled Salmonella by flow cytometry using the FACScalibur (BD Biosciences).

Statistical analysis

Survival of groups of mice was compared by the Kaplan-Meyer log-rank test. Differences in concentrations of cytokines and in organ counts of the microorganisms were analyzed by the Mann-Whitney U test. Differences were considered significant at P<.05. All the experiments were at least performed in duplicate.

Results

Outcome of Salmonella infection in LDLR−/− mice

The total cholesterol concentrations were significantly higher in the uninfected LDLR−/− mice than in their wild-type littermates (9.6±1.1 mmol/L vs. 2.3±0.5 mmol/L). After i.v. infection with 10⁴ cfu of S. typhimurium, only 5% of the LDLR−/− mice died, whereas the mortality of control LDLR+/+ was 100% within 12 days of infection (P<.001; Fig. 1). A similar difference in mortality was apparent when mice were infected intraperitoneally with S. typhimurium (10% mortality in LDLR−/− mice, vs. 100% mortality in control LDLR+/+ mice, p<0.01). The reduced mortality to infection in LDLR−/− mice was accompanied by a markedly reduced bacterial load in the organs (Fig. 2). On day 7 of infection, the differences between the control and LDLR−/− mice approached 10,000-fold (P<.001; Fig. 2).

Intracellular killing of Salmonella by cells from LDLR+/+ and LDLR−/− mice in vitro

The numbers of Salmonella CFU phagocytized by neutrophils and macrophages of LDLR−/− and LDLR+/+ mice were similar (Fig. 3A). In addition, the intracellular killing assay demonstrated that neutrophils and macrophages of LDLR−/− mice and LDLR+/+ mice did not differ in their ability to kill S. typhimurium intracellularly (Fig. 3B). The killing rate did not differ when lipoprotein-rich serum of LDLR−/− mice was coincubated with LDLR+/+ control macrophages, and likewise, serum from LDLR+/+ mice did not affect the killing of Salmonella by LDLR−/− macrophages (not shown).

Circulating cytokines during Salmonella infection

On day 1, cytokine concentrations in all samples were under the detection limit. No detectable concentrations of IL-1β (<20 pg/ml) were found at any time point during the infection. On day 3, IL-1α and TNFα were under the detection limit in LDLR−/− mice, while TNFα concentrations tended to be slightly higher (45±10 pg/ml) in LDLR+/+ mice (n.s.). On day 7, circulating concentrations of IL-1α and TNFα were significantly higher in LDLR+/+ than in LDLR−/− mice: 95±63 pg/ml vs 30±10 pg/ml for IL-1α (P<0.02) and 1140±290 pg/ml vs 43±26 pg/ml for TNFα (P<0.01) (Fig. 4A). These differences were most likely due to the greater amounts of Salmonella in the LDLR+/+ mice, leading to increased cytokine stimulation.

Figure 2. Outgrowth of S. typhimurium in the organs of LDLR+/+ and LDLR−/− mice. Outgrowth of S. typhimurium in the liver, spleen and bone marrow of LDLR−/− and control (LDLR+/+) C57Bl/6J mice after i.v. injection of 10^⁷ cfu. Each point represents the mean±SD for at least 10 animals. Significant differences between LDLR−/− and LDLR+/+ mice are indicated (*, P<0.01; ***, P<0.001; Mann-Whitney U test). doi:10.1371/journal.pone.0004237.g002
Inhibition of the interaction of Salmonella with monocytes and endothelial cells by lipoproteins

Because lipoproteins are known to bind and neutralize LPS, we preincubated Salmonella LPS and heat-killed whole Salmonella bacteria with LDL at various concentrations, and subsequently stimulated normal (LDLR+/+) macrophages for cytokine production. As shown in Fig. 6B, TNFα production induced by S. typhimurium is reduced in the presence of elevated LDL concentrations. To assess the effect of lipoproteins on the attachment of Salmonella to endothelial cells, FITC-labelled S. typhimurium was preincubated with LDL. Their attachment to endothelial cells was significantly reduced compared to that of Salmonella preincubated with lipoprotein-free plasma, as shown by both reduction of the percentage of cells binding Salmonella (97% cells bound LDLP-Salmonella, whereas only 82% cells bound LDL-Salmonella), as well as the mean fluorescence intensity per cell (41% reduction, from 56 to 33 conventional units) (see also Fig. 6C).

Protection against organ invasion by Salmonella in LDLR−/− mice

To investigate whether hyperlipoproteinemia influences the early organ invasion from the bloodstream by Salmonella, we determined the early distribution of the microorganisms after i.v. injection of $10^5$ S. typhimurium cfu. Blood from LDLR−/− mice contained significantly more Salmonella cfu than that of LDLR+/+ mice 30 minutes after injection ($P<0.01$), but an efficient elimination of the microorganisms occurred during the next 6 hours (Fig. 7A). The numbers of cfu in the liver and spleen were 70–80% lower in LDLR−/− mice than those in LDLR+/+ mice at 30 minutes after injection, and remained lower throughout the experiment, the difference between mouse strains being significant at 30, 60, 120 and 360 minutes for the liver (Fig. 7B, $P<0.05$) and at 30 and 60 minutes for the spleen (Fig. 7C, $P<0.05$).

Discussion

In the present study, we demonstrate that hyperlipoproteinemic mice are resistant against S. typhimurium infection. The protection was not due to the absence of the LDLR in the knock-out mouse strain, but to a direct effect of hyperlipoproteinemia. The beneficial effect of lipoproteins was exerted by blocking the interaction of Salmonella with host cells, including endothelial cells and monocytes, which led to inhibition of organ invasion. This resulted in an altered distribution of the microorganism to the organs of the host, and increased survival.

It has previously been shown that lipoproteins bind and neutralize LPS, with beneficial effects in Gram-negative infections [5–8]. As S. typhimurium is an LPS-containing Gram-negative bacterium, and Salmonella LPS plays a crucial role in cytokine stimulation [17] and induction of mortality in vivo [18], an
improved survival of LDLR−/− mice during systemic S. typhimurium infection would have been expected. Indeed, LDLR−/− mice were less susceptible to S. typhimurium infection, but this was not due to blunted cytokine production, as we observed undetectable or low cytokine circulating concentrations during the infection in the LDLR−/− and LDLR+/+ mice. In addition, the expression of cytokine mRNA was similar in the organs of LDLR−/− and LDLR+/+ mice. Thus, the cytokine response is not responsible for the mortality due to systemic S. typhimurium infection in this model, and the major difference is the almost complete absence of Salmonella in the organs of the LDLR−/− mice.

The low circulating cytokine response during systemic S. typhimurium infection may be attributed to the facultative intracellular nature of the organism. It should be noted that at later time points during the infection, the LDLR+/+ mice...
Figure 7. Hyperlipoproteinemia inhibits organ invasion by *S. typhimurium*. Distribution of *S. typhimurium* to the blood (cfu/ml), the liver (cfu/organ), and the spleen (cfu/organ) at various time points after i.v. injection of $10^5$ *Salmonella* cfu. Each point represents the mean±SD for at least 5 animals. Significant differences between LDLR−/− and LDLR+/+ mice are indicated (*p<0.05; **p<0.01; Mann-Whitney U test).

doi:10.1371/journal.pone.0004237.g007
exhibited a greater cytokine response than did LDLR<sup>−/−</sup> mice, and this is likely due to the 1000-fold greater bacterial burden in the control mice, leading to substantial stimulation of the host response.

We hypothesized that the decreased bacterial growth in the LDLR<sup>−/−</sup> mice may be a result of enhanced phagocytosis and intracellular killing of *Salmonella* organisms in these mice. However, this proved not to be the case; phagocytosis and subsequent intracellular killing of the organisms by both neutrophils and macrophages did not differ between LDLR<sup>−/−</sup> and LDLR<sup>+/+</sup> mice. Another reason for protection could have been the absence of the LDL receptor in the knock-out mice, *Toxoplasma gondii* is known to use the host LDLR for cholesterol acquisition [19], whereas penetration of cells by *Pseudomonas* exotoxin A is mediated through LDLR-related protein [20,21]. Thus, use of the LDLR by *Salmonella* during organ invasion could be envisaged, but was ruled out by the observation that hyperlipoproteinemic ApoE<sup>−/−</sup> mice, which have an intact LDLR, also were resistant to *Salmonella* infection. In addition, LDL affected the adhesion of *S. typhimurium* to LDLR-bearing endothelial cells. This demonstrates that elevated lipoprotein concentrations, and not the lack of LDLR itself, are responsible from the resistance of mice against *Salmonella* infection.

Theoretically, there are several mechanisms that could account for the beneficial effects of the lipoproteins on *Salmonella* infection. Firstly, a direct effect of lipoproteins on the growth of *Salmonella* could be envisaged. In this respect, it is of interest that HDL has been found to be cidal against *Trypanosoma cruzi* [20]. However, the growth of *Salmonella* was similar in the plasma of LDLR<sup>−/−</sup>, ApoE<sup>−/−</sup> and control mice, excluding a direct antimicrobial effect of LDL. Secondly, lipoproteins may interact with *Salmonella*, putatively with its LPS component, and thus block bacterial binding and internalization by host cells. As LPS is crucial for the internalization of *Salmonella* [22], blocking the interaction between LPS and host cells may prevent subsequent tissue invasion. Indeed, preincubation of *Salmonella* with LDL led to reduced cytokine production, demonstrating that lipoproteins are able to inhibit the interaction of *Salmonella* with monocytes. Even more relevant for tissue invasion, preincubation of *Salmonella* with LDL significantly reduced its attachment to endothelial cells. This protective mechanism in which lipoproteins block *Salmonella* interaction with endothelial cells by their blockade of LPS represents the same type of mechanism as previously shown by the blockade of MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) of *Staphylococcus* by naturally occurring antibodies, resulting in the inhibition of staphylococcal adhesion to endothelial cell and reduced tissue invasion [23].

Interaction of *Salmonella* with host cells likely is an important early step in the pathogenesis of invasive infection. The ability to infect tissue macrophages has been described as an invasive trait of *Salmonella* organisms in these mice. However, the blockade of MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) of *Staphylococcus* by naturally occurring antibodies, resulting in the inhibition of staphylococcal adhesion to endothelial cell and reduced tissue invasion [23].

**References**


**Acknowledgments**

The authors thank Prof. Jaap van Dissel (Leiden University Medical Center) and Dr Pierre Demacker for advice, and Ineke Verschueren for assistance with the animal experiments and cytokine assays.

**Author Contributions**

Conceived and designed the experiments: MGN LAJ MK JWMVdM BJK. Performed the experiments: MGN MK FW AFHS. Analyzed the data: MGN LAJ MK AFHS. Contributed reagents/materials/analysis tools: AFHS. Wrote the paper: MGN LAJ MK AFHS JWMVdM BJK.


