Lipids, inflammation and atherosclerosis

Stitzinger, M.

Chapter 5

SCAVENGER RECEPTOR BI MODULATES LPS INDUCED TNF-α PRODUCTION AND FORMS A HOST DEFENSE MECHANISM AGAINST INFLAMMATION

Miranda Stitzinger[1], Paula de Vos[1], Reeni B. Hildebrand[1], Dan Ye[1], Edwin Van Amersfoort[1], Menno Hoekstra[1], Yuemang Yao[2], Domenico Praticò[2], Theo J.C. Van Berkel[1], Miranda Van Eck[1] and Johan Kuiper[1]
[1]Division of Biopharmaceutics, LACDR, Leiden University, The Netherlands
[2]Department of Pharmacology, University of Pennsylvania, USA

Submitted for publication

ABSTRACT

LPS is a major constituent of the outer membrane of Gram-negative bacteria and stimulates monocytes and macrophages to produce high levels of pro-inflammatory cytokines, leading to cytotoxic effects, organ failure and eventually death. Scavenger receptors such as SR-A and SR-BI are involved in the metabolism of LPS and recently, it was demonstrated that SR-BI can prevent LPS induced death. In the current study, we challenged SR-BI deficient and wild-type mice with a sublethal dose of LPS and determined the induction of TNF-α in the circulation. SR-BI deficient mice expressed significantly higher levels of TNF-α in the circulation upon stimulation with LPS. Injection with radioactively labeled LPS demonstrated that LPS is rapidly cleared from serum, whereby LPS concentrations remained slightly higher in SR-BI deficient mice. SR-BI deficient mice display higher oxidative stress levels than wild-type mice. Treatment with the HDL-lowering anti-oxidant probucol reduced oxidative stress levels in SR-BI deficient mice to similar levels as in SR-BI wild-type mice. However, SR-BI deficient mice treated with probucol still responded to LPS with a higher TNF-α production than wild-type mice, indicating that the increased oxidative stress status of SR-BI deficient mice is not responsible for the increased TNF-α production upon LPS challenge. Although macrophage content in the liver was the same for both types of mice, these macrophages appeared to be more activated in SR-BI deficient mice, as determined by increased gene expression of MARCO, a scavenger receptor that is induced by inflammatory responses. The higher TNF-α response of the SR-BI deficient mice to LPS was also reflected in the *ex vivo* response of blood derived monocytes to LPS. These results show that the increased sensitivity of SR-BI deficient mice to LPS may relate to the higher initial activation status of these mice, indicating that SR-BI is important for a general host defense mechanism against inflammation.

INTRODUCTION

Sepsis is an important cause of death, which is induced by infections and Gram-negative bacteria contribute largely to the occurrence of sepsis[1,2]. Lipopolysaccharide (LPS) constitutes the outer membrane of Gram-negative bacteria and is responsible for the evocation of an immune response of monocytes and macrophages. In response to LPS, macrophages produce cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6[2-5]. Although cytokine production is important for the efficient control of growth and eradication of invading pathogens, high concentrations of these cytokines can lead to cytotoxic effects, organ failure and eventually death[2,6,7].
The LPS induced cytokine response primarily involves Toll-like receptor (TLR)4 and plasma membrane CD14, which initiate downstream signaling to NF-κB followed by activation of LPS responsive genes[8,9]. As a result, CD14 deficient and TLR4 deficient animals are resistant to endotoxic shock and infection by Gram-negative organisms[10,11]. In addition, LPS uptake, clearance, and catabolism are mediated by the family of scavenger receptors[12-14]. Scavenger receptors belong to a family of large structurally unrelated receptor proteins functionally defined by their ability to recognize modified low-density lipoproteins. Uptake of modified lipoproteins via scavenger receptors leads to foam cell formation, a hallmark of atherosclerosis[15,16]. In contrast to CD14 and TLR4, macrophage scavenger receptors have been implicated in the protection against LPS. Class A scavenger receptor (SR-A), which is expressed by activated macrophages, protects the host against infection with Listeria monocytogenes or herpes simplex virus type-1[17] as well as lethal endotoxic shock[14]. Moreover, phagocytosis of meningococci is mediated almost exclusively via SR-A[18]. Another important member of the scavenger receptor superfamily is scavenger receptor class B, type I (SR-BI). SR-BI is a prominent regulator of high-density lipoprotein (HDL) metabolism and mediates the selective uptake of cholesteryl esters from HDL without internalization of the HDL particle[19]. Selective disruption of SR-BI in mice results in increased plasma cholesterol levels due to the accumulation of large cholesteryl ester-rich HDL and is associated with an increased susceptibility to atherosclerosis[20,21]. Interestingly, SR-BI mRNA and/or protein levels are downregulated by LPS in liver, monocytes/macrophages and in colon carcinoma cells[22-24]. In addition, the human homologue of SR-BI, CLA-1, has been shown to be able to bind LPS and mediate internalization[25]. Furthermore, targeting of SR-BI by synthetic amphipathic α-helical-containing peptides blocks LPS uptake and LPS induced pro-inflammatory cytokine responses in THP-1 monocyte cells[26]. Recently, in studies using SR-BI deficient mice, Li *et al* showed that SR-BI prevents LPS-induced death, probably via inhibiting nitric oxide induced oxidative stress[27]. In the current study, we investigated the role of SR-BI in the response to a sublethal dose of LPS using SR-BI deficient and wild-type mice. Furthermore, we treated these mice with the HDL lowering anti-oxidant probucol in order to evaluate the relative importance of HDL levels and the presence of SR-BI in the inflammatory status of the mice.

MATERIALS AND METHODS

Animals

SR-BI deficient mice were kindly provided by Dr. M. Krieger (Massachusetts Institute of Technology, Boston, USA). Heterozygous SR-BI deficient mice were crossbred to generate wild-type and homozygous SR-BI deficient progeny. The offspring of the mice was analysed for presence of targeted and wild-type SR-BI alleles by polymerase chain reaction (PCR), as described by Van Eck *et al*[28]. Mice were fed a sterilized regular chow powder diet (RM3, Special Diet Services, Witham) or, if stated, chow powder supplemented with 0.5% (w/w) probucol (MP Biomedicals). Animal experiments were performed at the Gorlaeus Laboratories of the Leiden/Amsterdam Center for Drug Research in accordance with the national laws. All experimental protocols were approved by the Ethics Committee for Animal Experiments of the Leiden University.

TNF-α response

In vivo: Mice were intravenously injected with 50 µg/kg LPS from Salmonella minnesota R595 (List Biological Laboratories Inc) into the tail vein. Blood samples were collected after 30, 60, 90, 120 and 180 minutes and serum TNF-α levels were determined by ELISA (OptEIA kit, Pharmingen).

Ex vivo: Whole blood was obtained by tail vein transsection and diluted 25 times in DMEM culture medium supplemented with 0.2% bovine serum albumin (BSA), 100 IU/ml penicillin and 100 µg/ml streptomycin (P/S) and 4 mM L-glutamine, which contained various concentrations of LPS. After overnight incubation at 37°C, supernatant was collected and analysed for TNF-α content by ELISA.

Radiolabeling of LPS

LPS was iodinated according to the method of Ulevitch[29]. Briefly, 1 mg of LPS was incubated with 9.2 mg methyl-4-hydroxybenzimidate hydrochloride (Fluka Chemie) for 18 hrs at 37°C in 50 mM borate buffer pH 8.5. After thorough dialysis against phosphate buffered saline (PBS) pH 7.4 and finally PBS pH 8, half of the volume was added to a solution containing 10 µl of 4 mg/ml chloramine T (Merck) in borate buffer and 10 µl (0.25 mCi) $Na^{125}I$ (Amersham) in 0.1 M NaOH, which were pre-incubated for 1 min. The solution was mixed, and incubated for 1 min at room temperature (RT), after which 50 µl of 1.6 µg/ml KI in borate buffer was added. The reaction mixture was left for 30 min at RT, after which 10 µl of 4 mg/ml NaS_2O_3 in borate buffer was added to stop the reaction. The iodinated LPS (^{125}I-LPS) was dialysed extensively against PBS pH 7.4 and stored at 4°C. The concentration of ^{125}I-LPS was determined by the KDO assay as described by Karkhanis *et al*[30].

Serum clearance and liver uptake of ^{125}I-LPS

Mice were anesthetized and the abdomen was opened. An amount of 130 µg/kg ^{125}I-LPS in PBS was injected into the *inferior vena cava*. Blood samples were drawn from the *vena cava* and liver lobules were tied off at indicated time points. After 30 minutes, mice were sacrificed and the liver

was removed, weighed and counted for radioactivity. Blood samples were allowed to clot and serum was counted for radioactivity. The amounts of ^{125}I-LPS in liver and serum were expressed as percentage of injected dose. The liver levels were corrected for serum content of the tissue.

Serum cholesterol

Serum concentrations of total cholesterol were determined by enzymatic colorimetric assays with 0.025 U/ml cholesterol oxidase (Sigma), 0.065 U/ml peroxidase (Roche Diagnostics, Mannheim, Germany), and 15 µg/ml cholesteryl esterase (Roche Diagnostics) in reaction buffer (1.0 KPi buffer, pH=7.7 containing 0.01 M phenol, 1 mM 4-amino-antipyrine, 1% (v/v) polyoxyethylene-9-laurylether, and 7.5% (v/v) methanol). The cholesterol distribution over the different lipoproteins in serum was analysed by fractionation of 30 µl serum using a Superose 6 column (3.2×300 mm, Smart-system, Pharmacia). Total cholesterol content of the effluent was determined using enzymatic colorimetric assays as described above.

Oxidation status

Urinary, EDTA-anticogulated plasma, and liver levels of the isoprostane iPF2α-VI were measured by gas chromatography-mass spectrometry (GC/MS) as described previously[31,32]. Urine was collected during a 24 hour period. Blood samples were collected, centrifuged at 7,000 rpm for 10 min, and plasma was separated and stored at –80°C until analysis. Samples were spiked with a known amount of internal standard, extracted and purified by thin-layer chromatography, and analysed by negative ion chemical ionization GC/MS. Liver samples were minced and homogenized, and total lipids were extracted with ice-cold Folch solution (chloroform/methanol; 2:1, v/v). Next, base hydrolysis was performed using 15% KOH at 45°C for 1 hour to measure total levels of iPF2α-VI using an ion chemical ionization GC/MS assay, as described above.

^{125}I-LPS binding

Thioglycollate-elicited peritoneal macrophages were suspended in DMEM culture medium supplemented with 2% BSA, P/S and 4 mM L-glutamine and incubated with 25-2000 ng/ml ^{125}I-LPS in a total volume of 350 µl. After 2 hours of incubation at 4°C on a circulating laboratory shaker (150 rpm), the cells were washed with isotonic Tris.HCl (10 mM) buffered saline containing 5 mM $CaCl_2$ (pH 7.4): twice with buffer containing 0.2% BSA, once with buffer without BSA. Cells were lysed in 0.1 M NaOH and radioactivity was counted in a Packard gamma counter. Protein content was determined using the Lowry method.

Table 1: Primers for quantitative real-time PCR analysis

Gene	GenBank Accession	Forward primer	Reverse Primer
β-actin	X15267	GGACCCGAGAAGACCTCCTT	GCACATCACTCAGAATTTCAATGG
CD14	NM009841	TGGGCGAGAGAGGACTGATCT	TACGCAGCGCTAAAACTTGGA
CD68	NM009853	CCTCCACCCTCGCCTAGTC	TTGGGTATAGGATTCGGATTTGA
GADPH	NM008084	TCCATGACAACTTTGGCATTG	TCACGCCACAGCTTTCCA
HPRT	J00423	TTGCTCGAGATGTCATGAAGGA	AGCAGGTCAGCAAAGAACTTATAG
MARCO	MMU18424	AAAGGGTCAAAAAGGCGAATCT	AACTTCAGCTCGGCCTCTGTT
TLR4	NM021297	CATGGAACACATGGCTGCTAA	GTAATTCATACCCCTGGAAAGGAA

Gene expression
Thioglycollate-elicited peritoneal macrophages and livers were isolated and lysed using TriZol® reagent (Invitrogen Life technologies). RNA was isolated from cell lysates according to manufacturer's instruction and reverse transcribed using RevertAid™ reverse transcriptase. Gene expression analysis was performed using real-time SYBR Green technology (Eurogentec) with the primers displayed in **Table 1**. Hypoxanthine-guanine phosphoribosyltransferase (HPRT), acidic ribosomal phosphoprotein P0 (36B4) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as standard housekeeping genes. Relative gene expression numbers were calculated by subtracting the threshold cycle number (Ct) of the target gene from the average Ct of housekeeping genes and raising 2 to the power of this difference. The average Ct of three housekeeping genes was used to exclude that changes in the relative expression were caused by variations in the separate housekeeping gene expressions. The average Ct values for housekeeping genes did not differ significantly between groups.

Immunohistochemistry
Macrophages were detected in livers by immunolocalization of the MOMA-2 macrophage marker. Cryosections (6 µm) of liver from SR-BI deficient and SR-BI wild-type mice were incubated with 1% H_2O_2 in methanol for 20 minutes to quench endogenous peroxidase, after which they were washed and incubated with pre-heated 0.025% (v/v) trypsin for 5 minutes. After washing, sections were incubated for 30 minutes at 37°C with blocking buffer consisting of 5% fat-free milk and 0.1% Triton-X100 in PBS. Subsequently, sections were incubated with a 1:50 dilution of plyclonal rat-anti-mouse MOMA-2 IgG2b (Research Diagnostics) in blocking buffer for 30 minutes at 37°C. After washing, the sections were exposed for 2 hours at RT to a 1:200 dilution of goat-anti-rat IgG, conjugated to horseradish peroxidase (Brunschwig) in blocking buffer. Thereafter, sections were extensively washed and MOMA-2 positive macrophages were visualized by incubation with Novared (Dakocytomation) for 5-10 minutes and counterstained with hematoxylin (Sigma) for cell nuclei.

RESULTS

TNF-α response
To investigate whether SR-BI affects *in vivo* TNF-α induction by LPS, SR-BI deficient mice and wild-type mice were injected with a sublethal dose of LPS (50 µg/kg). Blood samples were collected up to 3 hours after injection and serum TNF-α levels were determined using ELISA. Both types of mice showed an increase in TNF-α levels starting at 30 min after injection and peaking at 60-120 min after LPS injection (**Fig. 1A**). However, the TNF-α levels in serum of SR-BI deficient mice were significantly ($p<0.001$) higher than in serum of wild-type mice (**Fig. 1A**). In an *ex vivo* whole blood assay, TNF-α production by monocytes of both SR-BI deficient and wild-type mice in response to incubation with various concentrations of LPS was determined. SR-BI deficiency was found to significantly enhance TNF-α secretion induced by 10-1500 ng/ml LPS ($p<0.001$, **Fig. 1B**).

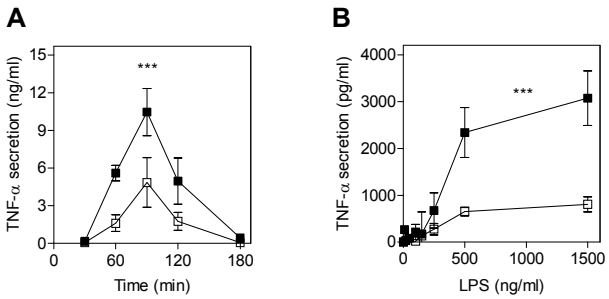

Fig. 1: LPS induced TNF-α production *in vivo* and *ex vivo*. A SR-BI deficient (filled squares) and wild-type (open squares) mice were intravenously injected with 50 µg/kg LPS, after which blood samples were collected and serum TNF-α levels were determined using ELISA. **B** Whole blood samples from SR-BI deficient (filled squares) and wild-type (open squares) mice were challenged overnight with different concentrations of LPS. TNF-α response was determined by measuring TNF-α levels in supernatant by ELISA. Values are expressed as mean±SEM. Significant difference compared to wild-type mice: *** $p<0.001$.

Serum clearance and liver uptake

To investigate the role of SR-BI in the removal of LPS from the blood circulation, we measured serum decay and liver uptake of intravenously injected ^{125}I-LPS. In both SR-BI deficient and wild-type mice, the serum decay of LPS was fast and after 15 minutes approximately 90% was cleared from the serum (**Fig. 2A**). However, in the first phase after injection of ^{125}I-LPS (2 minutes), serum concentration of LPS remained significantly ($p<0.05$) higher in SR-BI deficient mice compared to wild-type mice (**Fig. 2B**).

LPS is cleared from the blood mainly by the liver[33,34]. Therefore, we also determined the liver uptake of ^{125}I-LPS. At 2 minutes after injection, approximately 35% of the injected dose was recovered in liver, both in SR-BI deficient and wild-type mice. At 5 minutes after injection, the recovery of the injected dose in livers of both SR-BI wild-type and deficient mice reached approximately 45% of the injected dose and stayed at this level during the experiment (30 minutes, **Fig. 2C**). Thus, SR-BI deficiency does not affect the hepatic uptake of LPS.

Lipoproteins have been demonstrated to bind and, thereby, neutralize LPS[29,35,36]. As reported previously[37], serum total cholesterol levels are significantly higher ($p<0.001$, **Fig. 2D**) in SR-BI deficient mice (163±11 mg/dl) than in wild-type mice (77±4 mg/dl). The higher serum total cholesterol concentration in SR-BI deficient mice is due to an increase in the amount of cholesterol in abnormally large HDL particles ($p<0.001$, **Fig. 2E**). The increased levels of HDL in absence of SR-BI might explain the prolonged residence time of LPS in serum of SR-BI deficient mice, but not the higher TNF-α response after injection with LPS.

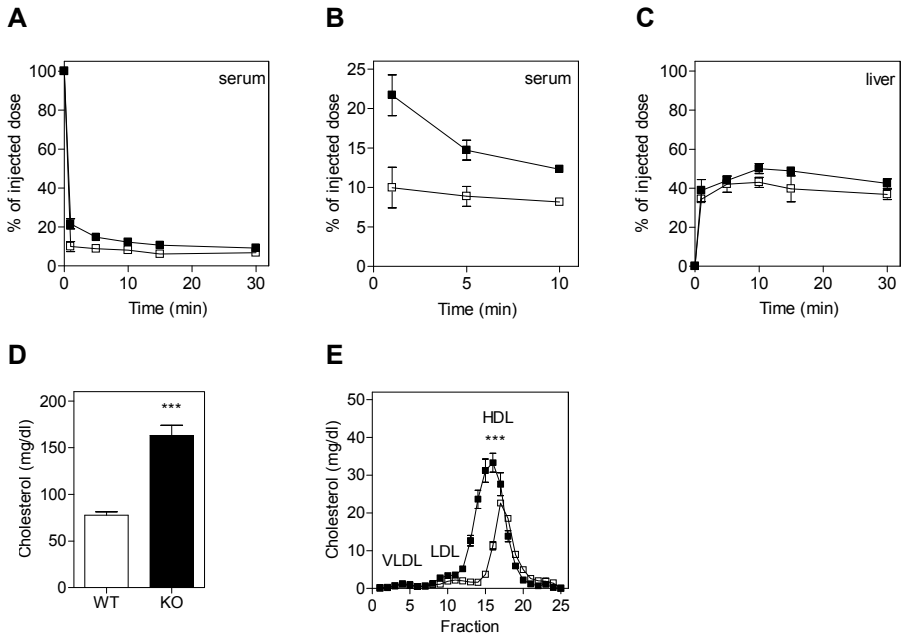

Fig. 2: Serum decay and liver uptake of LPS. SR-BI deficient (filled squares) and wild-type (open squares) mice were injected with ^{125}I-LPS via the *inferior vena cava*. At the indicated time points, radioactivity in serum (**A,B**) and liver (**C**) was determined. **D** Serum samples of SR-BI deficient (KO, filled) and wild-type (WT, open) were collected and analysed for total cholesterol content. **E** Serum samples were fractionated using a Superose 6 column and fractions were analysed for cholesterol content. Values are expressed as mean±SEM. Significant difference compared to wild-type mice: *** $p<0.001$

Oxidation

It had been reported that HDL can bind LPS and thereby neutralize the toxicity of LPS[38,39]. We studied whether treatment with the HDL lowering anti-oxidant probucol[40,41] was able to reduce the enhanced TNF-α response to LPS in SR-BI deficient mice. Therefore, SR-BI deficient and wild-type mice were fed a regular chow powder diet or chow powder supplemented with 0.5% (w/w) probucol for two weeks. As a sensitive and robust index of in vivo oxidative stress, we measured the generation of a major isoprostane, iPF2α-VI in liver, plasma and urine[32]. Significantly higher levels of iPF2α-VI were found in liver (2.09±0.29, $p<0.01$), plasma (1.38±0.12, $p<0.05$) and urine (1.99±0.14) of SR-BI deficient mice compared to wild-type mice fed a regular chow diet (**Fig. 3A-C**). Upon probucol treatment, iPF2α-VI levels were the same in liver and urine of SR-BI deficient and wild-type mice, but SR-BI deficient mice had lower iPF2α-VI levels in plasma (0.43±0.05, $p<0.05$) compared to wild-type mice (**Fig. 3A-C**).

Serum cholesterol levels also dropped after probucol treatment in both types of mice, but were still significantly higher ($p<0.001$, **Fig. 3D**) in SR-BI deficient mice (50±4 mg/dl) than those found in wild-type mice (15±2 mg/dl). The reduction in serum cholesterol levels was primarily reflected in decreased HDL cholesterol levels (**Fig. 3E**). In SR-BI deficient mice, probucol treatment reduced HDL cholesterol levels from 155±14 mg/dl to 52±4 mg/dl. HDL levels in the SR-BI deficient mice on the probucol diet thus dropped below the level in SR-BI wild-type mice on regular chow diet (75±2

mg/dl). However, HDL particles did remain abnormally large in size after probucol treatment.
To investigate the effect of LPS treatment under the reduced oxidant status, SR-BI deficient mice and control wild-type mice treated with probucol were injected with a sublethal dose of LPS (50 µg/kg) as described above. Both types of mice showed an increase in TNF-α levels starting at 30 min after injection and peaking at 60-120 min after LPS injection (**Fig. 3E**). Although probucol treatment normalized oxidative stress in the mice, TNF-α levels in serum of SR-BI deficient mice were still significantly higher ($p<0.001$) than in serum of wild-type mice (**Fig. 3E**) with levels even slightly higher as observed in the animals on regular chow diet.

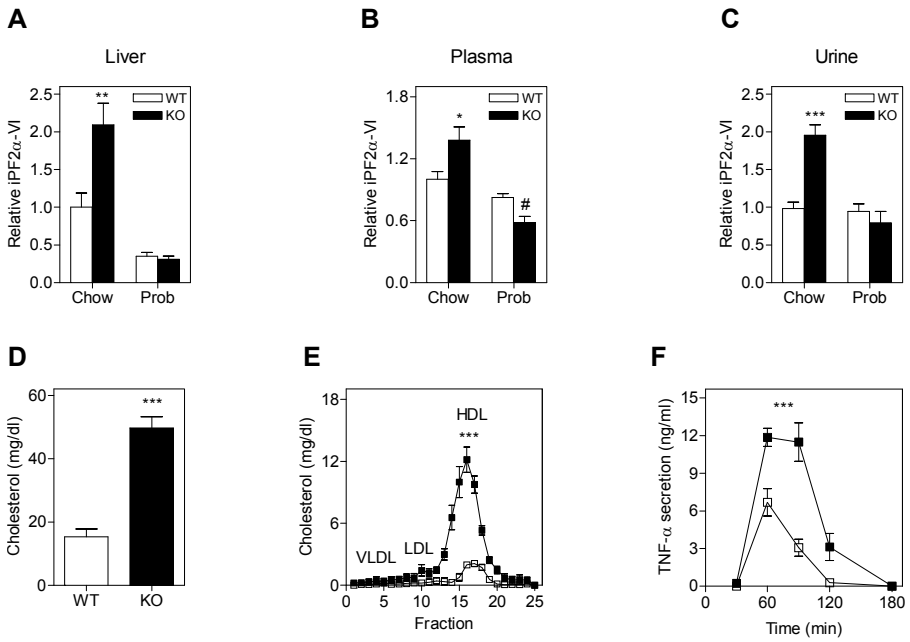

Fig. 3: Effect of treatment with the anti-oxidant probucol. SR-BI deficient and wild-type mice were fed a regular chow powder diet or chow powder supplemented with 0.5% (w/w) probucol for two weeks. The generation of a major isoprostane, iPF2α-VI, in **A** liver, **B** plasma and **C** urine was measured as an index of *in vivo* oxidative stress. **D** Serum samples of SR-BI deficient (KO) and SR-BI wild-type (WT) mice on the probucol diet were collected and analysed for total cholesterol content. **E** Serum samples of SR-BI deficient (filled squares) and SR-BI wild-type (open squares) mice on the probucol diet were fractionated using a Superose 6 column and fractions were analysed for cholesterol content. **F** SR-BI deficient (filled squares) and wild-type (open squares) mice on the probucol diet were intravenously injected with LPS, after which blood samples were collected and serum TNF-α levels were determined using ELISA. Values are expressed as mean±SEM. Significant difference compared to wild-type mice on a regular diet: * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Significant difference compared to wild-type mice on the probucol diet: # $p<0.05$.

Peritoneal and liver macrophages

Next, we investigated the effect of SR-BI deficiency on macrophage LPS binding and on macrophage expression of receptors for LPS. To quantitate LPS binding, thioglycollate-elicited peritoneal macrophages were incubated with 25-2000 ng/ml ^{125}I-LPS at 4°C for 2 hours. No significant difference was observed between LPS binding to SR-BI deficient and wild-type

peritoneal macrophages at the investigated ^{125}I-LPS concentrations (**Fig. 4A**), indicating that the observed higher TNF-α levels are not the result of altered binding of LPS to macrophages lacking SR-BI. In agreement, no significant difference in gene expression of LPS receptors CD14 and TLR4 was observed in SR-BI deficient macrophages compared to the expression in macrophages from wild-type mice (**Fig. 4B/C**).

Staining of livers of SR-BI deficient and wild-type mice for MOMA-2, a specific marker for macrophages, revealed that in both types of mice, the liver contained the same amount of macrophages, i.e. Kupffer cells (**Fig. 4D/E**). In addition, we isolated RNA from these livers for quantitative real-time PCR analysis of the gene expression of CD68, a general macrophage marker and of macrophage receptor with collagenous domain (MARCO), a scavenger receptor and an innate activation marker of macrophages. In agreement with the MOMA-2 immunohistochemical staining, no effect was observed of SR-BI deletion on CD68 expression (data not shown). The MARCO/CD68 ratio, however was significantly 4-fold higher in livers of SR-BI deficient mice than in liver of SR-BI wild-type mice ($p<0.001$, **Fig. 4F**), indicating that macrophages in SR-BI deficient livers are more activated than macrophages in SR-BI wild-type livers.

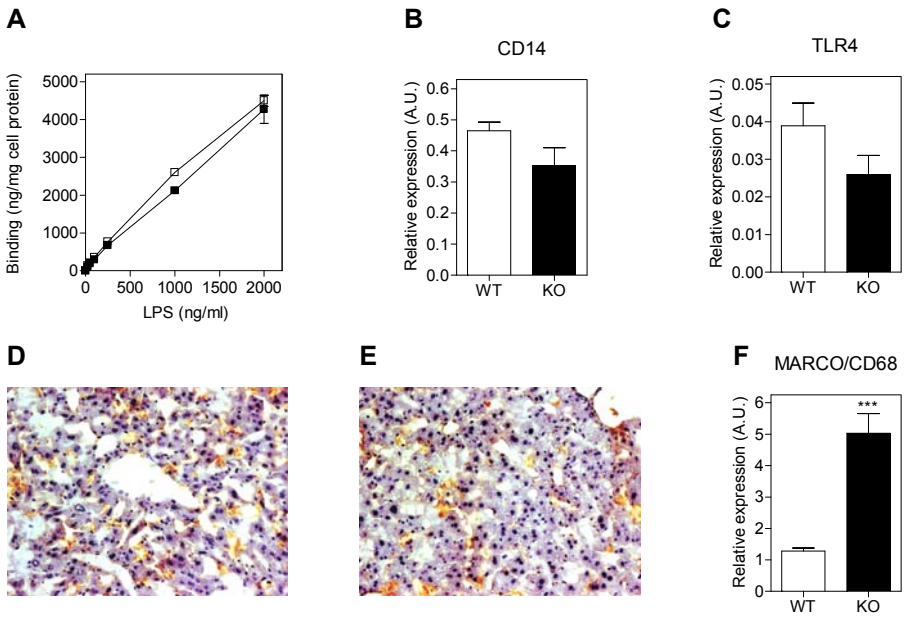

Fig. 4: Peritoneal and liver macrophages. **A** Peritoneal macrophages from SR-BI deficient (KO, filled squares) and SR-BI wild-type (WT, open squares) mice were incubated ^{125}I-LPS at 4°C for 2 hours, after which LPS binding was determined. RNA of peritoneal macrophages was isolated and quantitative real-time PCR analysis was used to determine gene expression of **B** CD14 and **C** TLR4. Livers of SR-BI wild-type (**D**) and SR-BI deficient (**E**) were stained for macrophage content using MOMA-2 and RNA of these livers was isolated for quantitative real-time PCR analysis of gene expression of MARCO compared to CD68 (**F**). Gene expression values are expressed as relative gene expression compared to housekeeping gene expression (arbitrary units) and all values expressed are as mean±SEM. Significant difference compared to wild-type mice: * $p<0.05$, *** $p<0.001$.

DISCUSSION

LPS is a major constituent of the outer membrane of Gram-negative bacteria and is one of the most potent inducers of inflammation[2-4]. LPS stimulates monocytes and macrophages to produce high levels of pro-inflammatory cytokines, leading to cytotoxic effects, organ failure and eventually death[2,5]. It has been demonstrated that LPS binding to TLR4 and CD14 plays a crucial in the initiation of intracellular signaling followed by activation of LPS responsive genes[8,9]. In addition, LPS uptake, clearance, and catabolism are mediated by members of the family of scavenger receptors, including SR-A and SR-BI[12-14]. Although SR-A is expressed by activated macrophages and protects the host against lethal endotoxic shock[14], LPS binding to SR-A has been demonstrated to have only a minor role in LPS clearance *in vivo*[13]. Moreover, phagocytosis of meningococci is mediated almost exclusively via SR-A, but LPS is not the ligand for SR-A on these microorganisms[18].

Interestingly, the human homologue of SR-BI, CLA-1, can mediate LPS binding and internalization[25] and *in vitro* targeting of SR-BI by synthetic peptides blocks LPS uptake and LPS induced pro-inflammatory cytokine responses in THP-1 monocyte cells[26]. In the current study, we show that SR-BI deficient mice display an increased response to a sublethal dose of LPS as evidenced by a larger induction of the secretion of TNF-α, one of the abundantly produced cytokines during infection and sepsis. These findings could explain the recent findings of Li *et al*[27], in which SR-BI expression prevented LPS induced death.

All lipoprotein classes are able to bind LPS and, thereby, attenuate the biological response to LPS[29,35,36]. High levels of HDL in SR-BI deficient mice could form an explanation for the slightly prolonged presence of LPS in serum of SR-BI deficient mice. The abnormally large HDL that accumulates in SR-BI deficient mice contains both apolipoprotein (apo)E and apoA-I, whereas HDL in SR-BI wild-type mice contains apoA-I and a small amount of apoA-II[37]. It has been shown that especially apolipoproteins, including apoA-I and apoE, are responsible for the protective effects of lipoproteins against LPS induced inflammatory effects[42,43]. However, despite high levels of apolipoprotein-rich HDL in SR-BI deficient mice, these mice are more susceptible to the LPS-induced TNF-α response in our study and LPS-induced death in the study of Li *et al*[27]. Recently, Thompson and Kitchens demonstrated that native HDL is able to suppress the inhibitory activity of LPS-binding protein (LBP), thereby augmenting the cellular response to LPS[43]. In our study, however, we show that reduction of HDL cholesterol levels in both SR-BI deficient and wild-type mice by probucol treatment does not influence TNF-α production in response to a challenge with a sublethal dose of LPS.

Li *et al* suggest that SR-BI prevents LPS induced death via inhibiting NO induced oxidative stress[27]. As a sensitive and robust index of *in* vivo oxidative stress, we measured the major isoprostane iPF2α-VI in liver, plasma and urine of SR-BI deficient and wild-type mice[31,32]. In untreated mice, oxidative stress levels were elevated in SR-BI deficient mice compared to those in wild-type mice, indicating that SR-BI protects against oxidative stress. On the other hand, we demonstrated that feeding SR-BI

deficient and wild-type mice a diet supplemented with probucol, an HDL lowering anti-oxidant, resulted in comparable oxidative stress levels in both types of mice, whereas the TNF-α response to LPS was still enhanced in SR-BI deficient mice and not lowered by the probucol treatment. Based on these results, we suggest that SR-BI plays an important role in maintaining low oxidative stress levels, but does not prevent the LPS induced effects via abrogation of oxidative stress. Interestingly, treatment with probucol does overcome infertility in SR-BI deficient mice[40] and prevents early coronary heart disease and death in mice deficient in both SR-BI and apoE[41]. Thus, although administration of probucol has a beneficiary impact on several pathologies in SR-BI deficient mice, it can not overcome the aggravated response to LPS of these mice.

The interaction of LPS with macrophages leading to activation of these cells plays a key role in the development of endotoxin shock. Kupffer cells, the macrophages of the liver, constitute the largest population of tissue macrophages in the body[44]. Depletion of Kupffer cells results in a marked decrease of the production of both TNF-α and protects against endotoxemia, indicating that Kupffer cells play a critical role in the development of endotoxemia[45]. In the current study we show that the uptake of LPS by the liver is not affected in SR-BI deficient mice, indicating that SR-BI is not involved in the initial uptake of LPS by the liver Kupffer cells. Subsequently, we investigated the effect of SR-BI deficiency on Kupffer content of the liver. No difference was observed in the amount of Kupffer cells between SR-BI deficient and wild-type mice. However, the ratio of MARCO/CD68 gene expression was 4-fold higher in SR-BI deficient mice. CD68 is a constitutively expressed macrophage marker and MARCO is highly activated by LPS in a time and dose dependent fashion[46]. Therefore, the higher ratio of MARCO/CD68 gene expression in SR-BI deficient mice indicates that SR-BI prevents innate activation of Kupffer cells. In addition, the *ex vivo* whole blood assay showed that also in circulating monocytes, SR-BI deficiency enhanced TNF-α secretion induced by LPS.

In conclusion, we demonstrate that SR-BI prevents TNF-α production induced by a sublethal dose of LPS. Although SR-BI is protective against oxidative stress, we proved that SR-BI does not exert its protective effect against LPS via reducing oxidative stress levels or via affecting HDL cholesterol levels. It is more likely that SR-BI inhibits LPS induced effects via attenuation of innate activation of circulating monocytes and Kupffer cells. These findings provide more insight in the beneficial presence of SR-BI, which is important for the understanding the role of SR-BI in inflammatory diseases, such as sepsis and atherosclerosis.

ACKNOWLEDGEMENTS

This research was supported by the Netherlands Heart Foundation (grants 2001B164, 2000T40 and 2001T41) and the National Institute of Health (grant AG-11542). Mieke Koenders is acknowledged for her assistance in obtaining liver cryosections.

REFERENCES

1. Glauser MP, Zanetti G, Baumgartner JD and Cohen J (1991) Septic shock: pathogenesis. *Lancet*. **338**: 732-736.
2. Van Amersfoort ES, Van Berkel TJ and Kuiper J (2003) Receptors, mediators, and mechanisms involved in bacterial sepsis and septic shock. *Clin Microbiol Rev*. **16**: 379-414.
3. Raetz CR (1990) Biochemistry of endotoxins. *Annu Rev Biochem*. **59**: 129-170.
4. Cohen J (2002) The immunopathogenesis of sepsis. *Nature*. **420**: 885-891.
5. Dobrovolskaia MA and Vogel SN (2002) Toll receptors, CD14, and macrophage activation and deactivation by LPS. *Microbes Infect*. **4**: 903-914.
6. Galanos C and Freudenberg MA (1993) Mechanisms of endotoxin shock and endotoxin hypersensitivity. *Immunobiology*. **187**: 346-356.
7. Dinarello CA (1996) Cytokines as mediators in the pathogenesis of septic shock. *Curr Top Microbiol Immunol*. **216**: 133-165.
8. Lien E, Means TK, Heine H, Yoshimura A, Kusumoto S, Fukase K, Fenton MJ, Oikawa M, Qureshi N, Monks B, Finberg RW, Ingalls RR and Golenbock DT (2000) Toll-like receptor 4 imparts ligand-specific recognition of bacterial lipopolysaccharide. *J Clin Invest*. **105**: 497-504.
9. Chow JC, Young DW, Golenbock DT, Christ WJ and Gusovsky F (1999) Toll-like receptor-4 mediates lipopolysaccharide-induced signal transduction. *J Biol Chem*. **274**: 10689-10692.
10. Haziot A, Ferrero E, Kontgen F, Hijiya N, Yamamoto S, Silver J, Stewart CL and Goyert SM (1996) Resistance to endotoxin shock and reduced dissemination of gram-negative bacteria in CD14-deficient mice. *Immunity*. **4**: 407-414.
11. Haziot A, Hijiya N, Gangloff SC, Silver J and Goyert SM (2001) Induction of a novel mechanism of accelerated bacterial clearance by lipopolysaccharide in CD14-deficient and Toll-like receptor 4-deficient mice. *J Immunol*. **166**: 1075-1078.
12. Hampton RY, Golenbock DT, Penman M, Krieger M and Raetz CR (1991) Recognition and plasma clearance of endotoxin by scavenger receptors. *Nature*. **352**: 342-344.
13. van Oosten M, van Amersfoort ES, van Berkel TJ and Kuiper J (2001) Scavenger receptor-like receptors for the binding of lipopolysaccharide and lipoteichoic acid to liver endothelial and Kupffer cells. *J Endotoxin Res*. **7**: 381-384.
14. Haworth R, Platt N, Keshav S, Hughes D, Darley E, Suzuki H, Kurihara Y, Kodama T and Gordon S (1997) The macrophage scavenger receptor type A is expressed by activated macrophages and protects the host against lethal endotoxic shock. *J Exp Med*. **186**: 1431-1439.
15. Mazzone T, Lopez C and Bergstraesser L (1987) Modification of very low density lipoproteins leads to macrophage scavenger receptor uptake and cholesteryl ester deposition. *Arteriosclerosis*. **7**: 191-196.
16. Steinberg D (1987) Lipoproteins and the pathogenesis of atherosclerosis. *Circulation*. **76**: 508-514.
17. Suzuki H, Kurihara Y, Takeya M, Kamada N, Kataoka M, Jishage K, Ueda O, Sakaguchi H, Higashi T, Suzuki T, Takashima Y, Kawabe Y, Cynshi O, Wada Y, Honda M, Kurihara H, Aburatani H, Doi T, Matsumoto A, Azuma S, Noda T, Toyoda Y, Itakura H, Yazaki Y, Kodama T and et al. (1997) A role for macrophage scavenger receptors in atherosclerosis and susceptibility to infection. *Nature*. **386**: 292-296.
18. Peiser L, De Winther MP, Makepeace K, Hollinshead M, Coull P, Plested J, Kodama T, Moxon ER and Gordon S (2002) The class A macrophage scavenger receptor is a major pattern recognition receptor for Neisseria meningitidis which is independent of lipopolysaccharide and not required for secretory responses. *Infect Immun*. **70**: 5346-5354.
19. Rigotti A, Trigatti B, Babitt J, Penman M, Xu S and Krieger M (1997) Scavenger receptor BI--a cell surface receptor for high density lipoprotein. *Curr Opin Lipidol*. **8**: 181-188.
20. Out R, Hoekstra M, Spijkers JA, Kruijt JK, van Eck M, Bos IS, Twisk J and Van Berkel TJ (2004) Scavenger receptor class B type I is solely responsible for the selective uptake of cholesteryl esters from HDL by the liver and the adrenals in mice. *J Lipid Res*. **45**: 2088-2095.

21. Covey SD, Krieger M, Wang W, Penman M and Trigatti BL (2003) Scavenger receptor class B type I-mediated protection against atherosclerosis in LDL receptor-negative mice involves its expression in bone marrow-derived cells. *Arterioscler Thromb Vasc Biol*. **23**: 1589-1594.
22. Khovidhunkit W, Moser AH, Shigenaga JK, Grunfeld C and Feingold KR (2001) Regulation of scavenger receptor class B type I in hamster liver and Hep3B cells by endotoxin and cytokines. *J Lipid Res*. **42**: 1636-1644.
23. Buechler C, Ritter M, Quoc CD, Agildere A and Schmitz G (1999) Lipopolysaccharide inhibits the expression of the scavenger receptor Cla-1 in human monocytes and macrophages. *Biochem Biophys Res Commun*. **262**: 251-254.
24. Peretti N, Delvin E, Sinnett D, Marcil V, Garofalo C and Levy E (2006) Asymmetrical regulation of scavenger receptor class B type I by apical and basolateral stimuli using Caco-2 cells. *J Cell Biochem*. .
25. Vishnyakova TG, Bocharov AV, Baranova IN, Chen Z, Remaley AT, Csako G, Eggerman TL and Patterson AP (2003) Binding and internalization of lipopolysaccharide by Cla-1, a human orthologue of rodent scavenger receptor B1. *J Biol Chem*. **278**: 22771-22780.
26. Bocharov AV, Baranova IN, Vishnyakova TG, Remaley AT, Csako G, Thomas F, Patterson AP and Eggerman TL (2004) Targeting of scavenger receptor class B type I by synthetic amphipathic alpha-helical-containing peptides blocks lipopolysaccharide (LPS) uptake and LPS-induced pro-inflammatory cytokine responses in THP-1 monocyte cells. *J Biol Chem*. **279**: 36072-36082.
27. Li XA, Guo L, Asmis R, Nikolova-Karakashian M and Smart EJ (2006) Scavenger receptor BI prevents nitric oxide-induced cytotoxicity and endotoxin-induced death. *Circ Res*. **98**: e60-65.
28. Van Eck M, Bos IS, Hildebrand RB, Van Rij BT and Van Berkel TJ (2004) Dual role for scavenger receptor class B, type I on bone marrow-derived cells in atherosclerotic lesion development. *Am J Pathol*. **165**: 785-794.
29. Ulevitch RJ (1978) The preparation and characterization of a radioiodinated bacterial lipopolysaccharide. *Immunochemistry*. **15**: 157-164.
30. Karkhanis YD, Zeltner JY, Jackson JJ and Carlo DJ (1978) A new and improved microassay to determine 2-keto-3-deoxyoctonate in lipopolysaccharide of Gram-negative bacteria. *Anal Biochem*. **85**: 595-601.
31. Pratico D, Tangirala RK, Rader DJ, Rokach J and FitzGerald GA (1998) Vitamin E suppresses isoprostane generation in vivo and reduces atherosclerosis in ApoE-deficient mice. *Nat Med*. **4**: 1189-1192.
32. Pratico D, Rokach J and Tangirala RK (1999) Brains of aged apolipoprotein E-deficient mice have increased levels of F2-isoprostanes, in vivo markers of lipid peroxidation. *J Neurochem*. **73**: 736-741.
33. Freudenberg MA, Freudenberg N and Galanos C (1982) Time course of cellular distribution of endotoxin in liver, lungs and kidneys of rats. *Br J Exp Pathol*. **63**: 56-65.
34. van Oosten M, van de Bilt E, van Berkel TJ and Kuiper J (1998) New scavenger receptor-like receptors for the binding of lipopolysaccharide to liver endothelial and Kupffer cells. *Infect Immun*. **66**: 5107-5112.
35. Feingold KR, Funk JL, Moser AH, Shigenaga JK, Rapp JH and Grunfeld C (1995) Role for circulating lipoproteins in protection from endotoxin toxicity. *Infect Immun*. **63**: 2041-2046.
36. Cavaillon JM, Fitting C, Haeffner-Cavaillon N, Kirsch SJ and Warren HS (1990) Cytokine response by monocytes and macrophages to free and lipoprotein-bound lipopolysaccharide. *Infect Immun*. **58**: 2375-2382.
37. Rigotti A, Trigatti BL, Penman M, Rayburn H, Herz J and Krieger M (1997) A targeted mutation in the murine gene encoding the high density lipoprotein (HDL) receptor scavenger receptor class B type I reveals its key role in HDL metabolism. *Proc Natl Acad Sci U S A*. **94**: 12610-12615.
38. Ulevitch RJ, Johnston AR and Weinstein DB (1979) New function for high density lipoproteins. Their participation in intravascular reactions of bacterial lipopolysaccharides. *J Clin Invest*. **64**: 1516-1524.
39. Wu A, Hinds CJ and Thiemermann C (2004) High-density lipoproteins in sepsis and septic shock: metabolism, actions, and therapeutic applications. *Shock*. **21**: 210-221.
40. Miettinen HE, Rayburn H and Krieger M (2001) Abnormal lipoprotein metabolism and reversible female infertility in HDL receptor (SR-BI)-deficient mice. *J Clin Invest*. **108**: 1717-1722.

41. Braun A, Zhang S, Miettinen HE, Ebrahim S, Holm TM, Vasile E, Post MJ, Yoerger DM, Picard MH, Krieger JL, Andrews NC, Simons M and Krieger M (2003) Probucol prevents early coronary heart disease and death in the high-density lipoprotein receptor SR-BI/apolipoprotein E double knockout mouse. *Proc Natl Acad Sci U S A*. **100**: 7283-7288.
42. Van Oosten M, Rensen PC, Van Amersfoort ES, Van Eck M, Van Dam AM, Breve JJ, Vogel T, Panet A, Van Berkel TJ and Kuiper J (2001) Apolipoprotein E protects against bacterial lipopolysaccharide-induced lethality. A new therapeutic approach to treat gram-negative sepsis. *J Biol Chem*. **276**: 8820-8824.
43. Flegel WA, Baumstark MW, Weinstock C, Berg A and Northoff H (1993) Prevention of endotoxin-induced monokine release by human low- and high-density lipoproteins and by apolipoprotein A-I. *Infect Immun*. **61**: 5140-5146.
44. Naito M, Hasegawa G, Ebe Y and Yamamoto T (2004) Differentiation and function of Kupffer cells. *Med Electron Microsc*. **37**: 16-28.
45. Hirose M, Nishikawa M, Qian W, Haque A, Mashimo M and Inoue M (2006) Mannose-conjugated alendronate selectively depletes Kupffer cells and inhibits endotoxemic shock in the mice. *Hepatol Res*. **36**: 3-10.
46. van der Laan LJ, Dopp EA, Haworth R, Pikkarainen T, Kangas M, Elomaa O, Dijkstra CD, Gordon S, Tryggvason K and Kraal G (1999) Regulation and functional involvement of macrophage scavenger receptor MARCO in clearance of bacteria in vivo. *J Immunol*. **162**: 939-947.