

Lipopolysaccharide Decreases Scavenger Receptor mRNA *In Vivo*

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ABSTRACT

Lipopolysaccharide (LPS) downregulates scavenger receptor (ScR) activity in cultured macrophages through release of tumor necrosis factor- α (TNF- α). However, LPS administration *in vivo* stimulates cytokine release from both macrophages and lymphocytes, the combined effects of which could alter ScR expression differently from TNF- α in isolation. To investigate whether LPS regulates ScR *in vivo*, 10 $\mu\text{g/g}$ was injected i.p. into Swiss Webster mice. Administration of LPS produced a profound decrease in hepatic ScR mRNA, with reductions of $74\% \pm 8\%$ at 2 h that returned to baseline levels by 6 h. Changes in ScR mRNA abundance coincided with changes in serum concentrations of TNF- α , which peaked at 2 h (1320 ± 309 pg/ml) and returned to preinjection concentrations at 4 h. Serum concentrations of interferon- γ (IFN- γ) did not increase until 4 h after injection of LPS. There was no effect on ScR mRNA abundance following LPS administration to LPS-resistant strains of mice, C3H/HeJ and IFN- γ receptor^{-/-}. The LPS-induced reduction in ScR mRNA in Swiss Webster mice was not sufficiently sustained to affect receptor function, as determined by the kinetics of [¹²⁵I]-acetylated LDL clearance from plasma. Therefore, as observed in cultured cells, LPS administration to mice decreases ScR mRNA despite the release of several cytokines *in vivo*.

INTRODUCTION

SCAVENGER RECEPTORS (ScR) ARE HYPOTHESIZED TO be involved in several disease processes, including atherosclerosis and host defense.^(1,2) The diversity of biologic roles stems from the unusually broad ligand specificity of this receptor type.⁽³⁾ ScR have been detected on a wide variety of cell types, including macrophages, fibroblasts,^(4,5) smooth muscle cells,⁽⁶⁾ and endothelial cells.⁽⁷⁾ The ScR present on macrophages are thought to account for the major biologic roles of this endocytic system.^(8,9)

ScR are downregulated by lipopolysaccharide (LPS) in cultured human monocyte-macrophages,⁽¹⁰⁾ mouse peritoneal macrophages,⁽¹¹⁾ and J774 cells,⁽¹²⁾ a mouse macrophage line. The LPS-induced downregulation of ScR in cultured macrophages is attributable to release of tumor necrosis factor (TNF- α) acting as an autocrine mediator.⁽¹³⁾ However, administration of LPS *in vivo* leads to release from cells of a wide variety of cytokines in addition to macrophages, including lymphocytes.⁽¹⁴⁾ Lymphocyte-conditioned media⁽¹⁵⁻¹⁸⁾ and specific lymphocyte-derived cytokines⁽¹⁹⁻²¹⁾ regulate ScR in cultured

macrophages. In particular, interferon- γ (IFN- γ) regulates ScR activity in cultured macrophages. Therefore, the administration of LPS to a whole animal could induce release of a spectrum of lymphocyte and macrophage cytokines, the combined effect of which may be at variance with that with a single cytokine on cultured cells.⁽²²⁾ A further potential confounding factor in extrapolating the effects observed in cell culture is that effects of LPS on ScR regulation have been evaluated at concentrations of endotoxin that would not be achievable *in vivo*.

Therefore, to study whether LPS regulates ScR *in vivo*, this material was injected into three strains of mice that have a range of responses to endotoxin. Relative abundance of ScR mRNA was determined in the liver of these strains. There is pronounced ScR activity in the liver that, in the mouse, is restricted to cells of macrophage origin.⁽²³⁾ In this study, the relative abundance of ScR mRNA in liver was compared with temporal changes in serum concentrations of TNF- α and IFN- γ , two major cytokines released by LPS that have been implicated in ScR regulation. The effects of acute administration of LPS on ScR function were also examined.

These experiments demonstrated that acute administration of

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LPS *in vivo* produces a pronounced, but transient, decrease in hepatic ScR mRNA that is mirrored by changes in serum concentrations of TNF- α . However, the decrease in ScR mRNA is not sufficiently sustained to decrease endocytic function. Despite data from cell culture studies to the contrary, marked increases in serum IFN- γ concentrations are not associated with downregulation of ScR mRNA.

MATERIALS AND METHODS

Animals

Swiss Webster mice were obtained from Charles River Laboratories. C3H/HeJ mice (endotoxin resistant)⁽²⁴⁾ were obtained from Jackson Laboratories (Bar Harbor, ME). IFN- γ receptor $-/-$ mice⁽²⁵⁾ were a gift from Dr. Robert D. Schreiber (Washington University). Mice were housed in specific pathogen-free rooms and fed a normal mouse diet (Purina Test Diets, Richmond, IN). All procedures were approved by the Washington University Animal Studies Committee.

Injection of LPS

LPS from *Escherichia coli* serotype 0111:B4 was obtained from Sigma Chemical Co. (St. Louis, MO). LPS (10 $\mu\text{g/g}$ in 0.9% saline) was injected i.p., and livers were removed from groups of five animals both before the injection and at intervals of up to 8 h and were then frozen in liquid nitrogen. Blood samples were drawn from the retroorbital sinus before liver removal, and the serum was stored at -80°C .

RNA Extraction

RNA was extracted by use of RNeasy B (Tel-Test, Friendswood, TX). Frozen livers were crushed in a stainless steel pestle and mortar at the temperature of liquid nitrogen. The purity and integrity of the RNA were confirmed spectrophotometrically ($A_{260}/A_{280} > 1.9$) and by agarose electrophoresis, respectively.

Synthesis of [^{32}P]-labeled cRNAs

Murine ScR cDNA fragments corresponding to a region common to both type I and type II receptors were synthesized from mouse liver RNA by reverse transcription (RT) followed by polymerase chain reaction (PCR) amplification (cDNA Cycle kit, Invitrogen, San Diego, CA). Sequences for mouse ScR were obtained from GeneBank (Accession No. L04274).⁽²⁶⁾ Oligonucleotide primers were 5'-GAACGAGAGGATGCT-GACTG (sense) and 3'-GTCATTGCACCTTTACTCTAAA (antisense), spanning a 302 base pair (bp) segment from bases 41 to 342. PCR products were gel purified (GlassMAX, BRL, Gaithersburg, MD), ligated into pGEM-T, and transformed into competent JM109 cells (Promega, Madison, WI). Plasmid DNA was purified with Wizard kits (Promega), and the size and orientation of inserts were confirmed by restriction mapping and sequencing (Sequenase, USB, Cleveland, OH). Plasmids were linearized with *Sa*I or *Nco*I. [^{32}P]CTP-labeled (Amersham, Arlington Heights, IL) sense and antisense RNAs were synthesized by *in vitro* transcription with either SP6 or T7 RNA polymerases (Promega, Madison, WI). Mouse β -actin cDNA was

obtained from Ambion (Austin, TX), and [^{32}P]-labeled cRNA was synthesized as described.

Ribonuclease protection assay

Assays were performed with RPA II kits (Ambion). Aliquots of liver RNA (50 μg) were hybridized overnight at 45°C with sense or antisense [^{32}P]cRNAs for both ScR and β -actin. Both RNase-treated and RNase-untreated [^{32}P]cRNAs and hybridized probes were run as controls. After digestion with RNase A/T1 and precipitation, protected fragments were heat denatured and separated on 6% urea/polyacrylamide gels. Autoradiography was performed with BioMax film (Eastman Kodak, Rochester, NY) at -80°C . Relative abundance of mRNA was determined by computerized densitometric scanning by use of Image-Pro Plus (Media Cybernetics, Silver Springs, MD). Changes in ScR abundance relative to β -actin were normalized to the ratio determined in mice not injected with LPS.

Assay of TNF- α and IFN- γ in serum

TNF- α and IFN- γ concentrations were determined by solid-phase sandwich enzyme-linked immunosorbent assays (catalog Nos. 3012 and 4022, respectively, Biosource, Camarillo, CA) according to the manufacturer's instructions. Samples from each of the time points in all three strains of mice were stored at -20°C and analyzed in a single assay.

Preparation and injection of radioiodinated acetylated low-density lipoprotein (AcLDL)

Human LDL ($d = 1.019\text{--}1.063\text{ g/ml}$) was isolated by ultracentrifugation with an L8-70 ultracentrifuge in a Ti-50.3 rotor (Beckman, Palo Alto, CA). Acetylated LDL (AcLDL) was prepared by incubation of acetic anhydride with LDL in the presence of sodium acetate and dialyzed extensively against saline (0.9%), as described previously.⁽²⁷⁾ Radioiodination of AcLDL was achieved with IodoBeads (Pierce Chemical Co., Rockford, IL) in the presence of [^{125}I] (Amersham). After fractionation through Sephadex G25 spin columns, radioactivity was $93\% \pm 3\%$ TCA precipitable, and specific radioactivities ranged from 1200 to 1500 cpm/ng protein. Protein concentrations were determined as described by Lowry et al.⁽²⁸⁾ with use of bovine serum albumin (BSA) as standard.

Mice anesthetized with metaphane administered by inhalation, followed by i.p. ketamine (90 mg/kg) and xylazine (13 mg/kg), were used to determine clearance of AcLDL from plasma. [^{125}I]AcLDL (5 μg in 50 μl of 0.9% saline) was injected via a tail vein. Blood was drawn at selected intervals from the retroorbital venous plexus into 50 μl heparinized capillary tubes, plasma was isolated by centrifugation, and TCA-precipitable radioactivity was determined. To determine the effect of LPS on the rate of clearance of [^{125}I]AcLDL from plasma, groups of mice were pretreated with LPS (10 $\mu\text{g/g}$) for 2 h. To determine the component of clearance attributable to ScR, mice were given i.v. fucoidan (300 μg) and [^{125}I]AcLDL simultaneously.

DNA sequence analyses

DNA sequences for ScR promoter regions were obtained from GeneBank and analyzed for the presence of putative cytokine

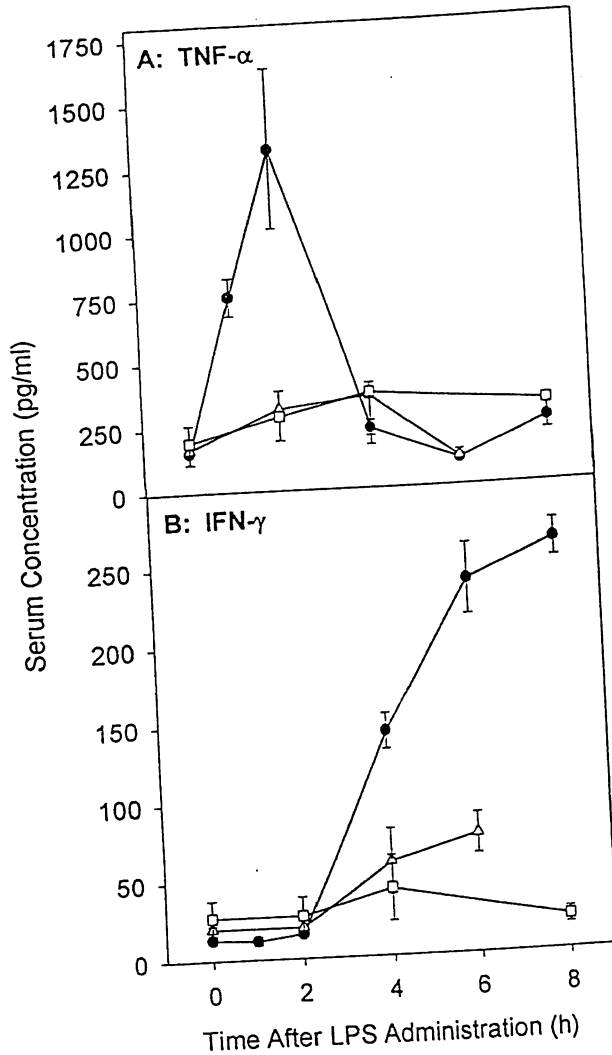


FIG. 2. Cytokine induction after i.p. injection of LPS into mice. (A) TNF- α concentrations in serum after administration of LPS. Swiss Webster mice (●) responded to LPS with a rapid rise in serum TNF- α concentrations, peaking at 2 h and then decreasing to preinjection concentrations. IFN- γ receptor^{-/-} mice (Δ) showed only a modest increase in concentrations of TNF- α after LPS administration, and there was no effect on concentrations in C3H/HeJ mice (□). (B) IFN- γ concentrations in serum after administration of LPS. In Swiss Webster mice (●), LPS did not result in increased serum concentration of IFN- γ concentrations until 4 h, and the plateau occurred at 6 h. Administration of LPS (10 μ g/g) resulted in small increases in serum concentrations of IFN- γ in IFN- γ receptor^{-/-} mice (Δ) and had no effect in C3H/HeJ mice (□). Points represent the mean of measurements on five animals and bars represent SEM.

DISCUSSION

Macrophage ScR activity is thought to have an important role in several disease processes, although factors that regulate the activity of this receptor *in vivo* have not been defined. Previous work in cultured macrophages demonstrated that LPS downregulates ScR. However, LPS stimulates a coordinated release of multiple cytokines from macrophages and lymphocytes *in vivo* such that findings in cultured cells may not provide in-

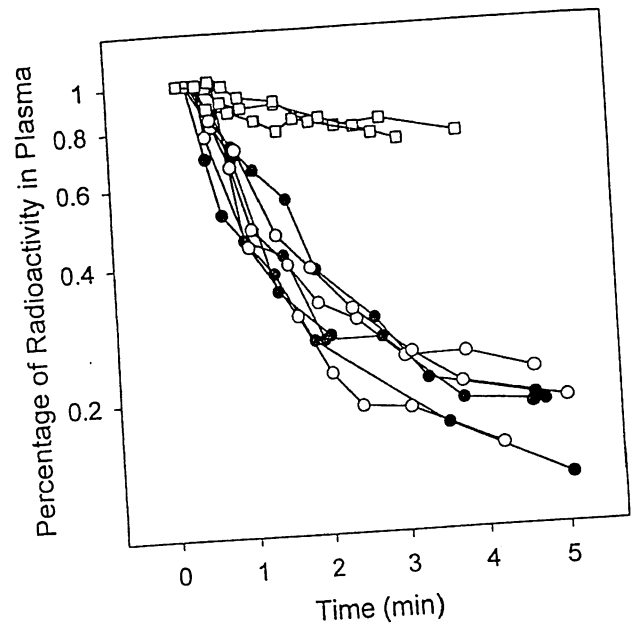


FIG. 3. ScR-mediated clearance of [¹²⁵I]AcLDL from plasma in Swiss Webster mice. [¹²⁵I]-labeled AcLDL (5 μ g protein, 50 μ l volume) injected into a lateral tail vein was cleared rapidly from plasma of Swiss Webster mice (●). Clearance of [¹²⁵I]AcLDL from plasma was almost completely inhibited by coinjection of fucoidan (300 μ g, □), indicating that a major component of AcLDL uptake was mediated by ScR. Clearance of [¹²⁵I]AcLDL from plasma was not altered by administration of LPS 2 h previously (○). Each curve represents data from a separate animal. Points represent individual determinations of TCA-precipitable plasma radioactivity at the times shown, normalized to the radioactivity measured at the earliest time.

sight into mechanisms operative *in vivo*. Our results demonstrate that acute administration of LPS produces a pronounced but transient decrease in ScR mRNA. Among the limited number of cytokines that it was feasible to quantify, serum concentrations of TNF- α had a close inverse relationship to temporal changes in ScR mRNA. In contrast, acute administration of LPS produced a delayed increase in serum concentrations of IFN- γ that was not associated with a decreased abundance of ScR mRNA.

Despite the release of several cytokines after administration of LPS to mice *in vivo*, the decreased ScR mRNA abundance in liver was consistent with effects of endotoxin on cultured macrophages. Cells of macrophage origin express most of the ScR protein in mouse hepatic tissue. Furthermore, the decrease in ScR mRNA after LPS administration correlated with increased serum concentrations of TNF- α . Although this correlation does not define a cause and effect relationship, it is again consistent with findings of studies using cultured macrophages. In contrast to effects noted in cultured macrophages, TNF- α increases ScR activity in smooth muscle cells.⁽³¹⁾ However, it is unlikely that this cell type contributes to the LPS-induced effects we observed in mouse hepatic tissue *in vivo*.

The specific molecular effects of TNF- α that lead to changes in ScR expression have not been defined. Binding of TNF- α to cell surface receptors can induce phosphorylation of the cytoplasmic inhibitory subunit I- κ B, allowing cytoplasmic NF- κ B

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to bind to enhancer sites on DNA and causing increased expression of several genes.⁽³²⁾ TNF- α may also suppress gene expression by inducing a nuclear protein that displaces the p50 homodimer of NF- κ B from a specific TNF- α inhibitory response element. Such response elements are found on several genes downregulated by TNF- α . Analysis of the promoter region of the human ScR gene^(33,34) revealed the presence of the sequence 5'-GGCGGCC-3', a putative TNF- α inhibitory response element commencing 642 bp downstream of the transcription start site. This sequence also occurs in the human thrombomodulin gene,⁽³⁵⁾ 147 bp downstream of the transcription start site, a gene that is also downregulated by TNF- α .⁽³⁶⁾ The presence of this sequence in the ScR gene may be a mechanism by which TNF- α reduces ScR transcription. Selected deletions of the ScR promoter region will enable elucidation of the importance of this element.

Injection of LPS into C3H/HeJ mice did not cause any change in either serum concentrations of TNF- α or hepatic mRNA abundance. Although this response is consistent with TNF- α release affecting ScR mRNA levels, this approach is hampered by the generalized unresponsiveness of C3H/HeJ mice to LPS. Knockout mice in which only one cytokine has been deleted are not useful in experiments to define a specific mechanism because the interdependence of cytokines prohibits identification of the effects of a single one. The importance of this relationship is exemplified in IFN- γ receptor^{-/-} mice, which are endotoxin resistant partly because of their inability to amplify the TNF- α response to LPS.⁽³⁷⁾ In wild-type mice, LPS stimulates macrophages to release TNF- α and IL-12,⁽³⁸⁾ and IL-12 stimulates natural killer (NK) cells to produce IFN- γ . IFN- γ then stimulates macrophages to release more TNF- α .⁽³⁹⁾ The interrelationship between the cytokines compromises the use of exogenous TNF- α or neutralizing antibodies to TNF- α in experimental models designed to identify individual effects of TNF- α and IFN- γ . Furthermore, the demonstrated lack of effect of LPS on ScR mRNA abundance in both C3H/HeJ and IFN- γ receptor^{-/-} mice indicates that there are no direct effects on ScR mRNA abundance. A direct effect is possible given that LPS is recognized by ScR.^(40,41)

Although a variety of responses of ScR to IFN- γ in cultured cells has been documented, the majority of studies have demonstrated downregulation. Therefore, a striking finding of the present study was the return of ScR mRNA abundance to preinjection levels during a phase of marked increases in serum concentrations of IFN- γ . Serum concentrations are not necessarily an indicator of local tissue concentrations of IFN- γ , since there could be a significant contribution from T lymphocytes and NK cells resident in tissues. However, normal hepatic tissue does not contain either of these cell types, and, therefore, the concentration of IFN- γ in the serum is probably representative of the cytokine concentrations at hepatic macrophages. IFN- γ has been shown to decrease ScR activity and mRNA in cultured macrophages of both murine and human origin.^(19,20) Indeed, the distribution of IFN- γ within human atherosclerotic lesions has been related to the presence of ScR protein.⁽⁴²⁾ T lymphocytes, a potential source of IFN- γ , have been detected within atherosclerotic lesions from humans,⁽⁴³⁾ rabbits,⁽⁴⁴⁾ and mice.⁽⁴⁵⁾ However, IFN- γ has also been demonstrated to have no effect over a wide range of concentrations.⁽⁴⁶⁾ In our own preliminary studies, the effects of IFN- γ on ScR activity var-

ied depending on culture conditions and macrophage origin (S.E. Roselaar, J.A. Comicelli, and A. Daugherty, unpublished observations). Another possible explanation for the lack of decrease in ScR mRNA in association with prominent increases in serum concentrations of IFN- γ is that the cytokine downregulates ScR *in vivo* but that other mediators released after LPS administration negate the inhibitory effects. Even if this is the case, the presence of IFN- γ would not be a requirement for ScR downregulation within atherosclerotic lesions.

The decrease in ScR mRNA that we observed was not associated with a decrease in receptor function. Since the majority of AcLDL is cleared from plasma by liver, the abundance of ScR protein in this tissue would affect the kinetics of removal of AcLDL. The lack of effect on ScR function despite a profound transient decrease in mRNA abundance for this receptor may reflect a protracted half-life of the protein. At present, the half-life of ScR protein is unknown, but functionally related proteins, such as LDL receptor, have a half-life of between 12 and 24 h.^(47,48) The absence of an effect of a transient decrease in ScR mRNA on a protein with a sustained half-life would not be surprising. However, in the setting of an atherosclerotic lesion, the finding of TNF- α by immunocytochemical analyses⁽⁴⁹⁻⁵²⁾ is consistent with a sustained presence of this cytokine that would presumably lead to decreased function. This supposition is supported by the recent demonstration of increased ScR protein in atherosclerotic lesions from mice deficient in the major TNF- α receptor p55.⁽⁵³⁾

In summary, we have demonstrated that administration of LPS leads to a profound but transient decrease in hepatic ScR mRNA *in vivo*. The decrease and subsequent return to preinjection abundance was temporally associated with changes in serum concentrations of TNF- α . Even in the presence of markedly elevated serum concentrations of IFN- γ , there was no decrease in ScR mRNA abundance.

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