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Lipopolysaccharide Induces Scavenger Receptor A Expression in Mouse Macrophages: A Divergent Response Relative to Human THP-1 Monocyte/Macrophages[1]

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Gene deletion studies indicate that the macrophage scavenger receptor A (SR-A) protects mice from LPS-induced endotoxemia. Paradoxically, cultured human monocyte-derived macrophages down-regulate SR-A expression when exposed to LPS. We found that human THP-1 monocyte/macrophages decrease SR-A expression in response to LPS independent of their differentiation status. In contrast, primary and elicited mouse peritoneal macrophages as well as the J774A.1 and RAW264.7 mouse macrophage lines increase SR-A expression in response to LPS. Exposure to LPS caused J774A.1 and RAW264.7 cells to increase SR-A transcripts by 3- and 5-fold, respectively. LPS caused a concomitant 3-fold increase in SR-A protein levels and increased cell membrane expression of the receptor. RAW264.7 cells increased SR-A transcript levels in response to LPS at concentrations as low as 1 ng/ml, and the response was saturated at 10 ng/ml. The LPS induction of SR-A transcripts required continual protein synthesis and began at 8 h, peaked by 16 h, and persisted for at least 48 h. LPS induction did not increase SR-A gene transcription or affect alternative transcript splicing, but mildly increased mature transcript stability and proceeded in the presence of actinomycin D. Finally, treatment of RAW264.7 cells with TNF-α did not induce SR-A transcript levels, indicating that a TNF-α autocrine/paracrine signaling mechanism alone is not sufficient to recapitulate the LPS induction of SR-A transcripts. The induction of SR-A expression by LPS-stimulated mouse macrophages is the opposite of the down-regulation of SR-A reported in human monocyte-derived macrophages and may have implications for the observed resistance mice show toward endotoxemia. ***The Journal of Immunology*****, 2000, 164: 2692–2700.**

Endotoxemia is classically associated with Gram-negative bacterial infections (1). During Gram-negative infections, a major portion of the host's inflammatory response is elicited by the bacterial cell wall component LPS, which activates a variety of immune cells, including tissue macrophages and circulating monocyte/macrophages. LPS-activated macrophages principally secrete the inflammatory cytokines TNF-α and IL-1. This cytokine secretion is thought to initiate uncontrolled cellular activation that leads to blood vessel damage, plasma leakage into tissues, hypotension, and organ failure.

Cell surface molecules termed scavenger receptors have been implicated in host defense through the clearance of LPS and other pathogenic molecules from the circulation (2). Of the known scavenger receptors, macrophage scavenger receptor A (SR-A)[3] has been most thoroughly characterized. SR-A was first cloned on the basis of its ability to bind and mediate the cellular uptake of modified LDL (3, 4). Subsequent studies showed that SR-A is a homotrimeric glycoprotein that has broad ligand specificity, with the capacity to bind a variety of polyanionic molecules (5, 6). LPS is among the pathogenic molecules recognized by SR-A, and it was found that the SR-A-mediated internalization of LPS leads to its degradation but does not induce cellular activation (7). Finally, in mice SR-A appears to protect against LPS-induced toxic shock, because SR-A knockout animals primed with Calmette-Guérin bacillus are more sensitive to the endotoxic effects of LPS (8).

Paradoxically, however, in human monocyte-derived macrophages LPS exposure decreases scavenger receptor activity and SR-A expression (9). This down-regulation in human macrophages has been attributed to TNF-α secretion, which is thought to destabilize SR-A mRNA through a paracrine/autocrine mechanism (10, 11). Additional differences in the transcriptional regulation of human and mouse SR-A expression have been suggested by studies of the proximal promoter elements of the SR-A gene in these two species (12, 13).

In the present study we examined the regulation of SR-A expression in mouse macrophage populations exposed to LPS. We confirmed that SR-A expression is down-regulated in a human monocyte-macrophage cell line exposed to LPS. In contrast, we found that SR-A expression was strongly up-regulated in mouse macrophages exposed to LPS. Our results indicate that the down-regulation of SR-A observed in human monocyte/macrophages is not a universal response of macrophages to LPS.

Materials and Methods

Reagents

The following reagents were purchased from the indicated vendors: cell culture grade *Escherichia coli* serotype 0111:B4 LPS, actinomycin D, Hoechst 33258, and cycloheximide (Sigma, St. Louis, MO); DMEM and RPMI cell culture medium (Life Technologies/BRL, Gaithersburg, MD); L-[U-^{14}C]leucine (303 mCi/mmol, 50 μCi/ml; Amersham, Arlington Heights, IL); [α-^{32}P]dCTP (3000 Ci/mmol) and [α-^{32}P]UTP (800 Ci/mmol; DuPont/NEN, Boston, MA); monoclonal 2F8 anti mouse SR-A Ab

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[3] Abbreviation used in this paper: SR-A, scavenger receptor A.

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(Serotec, Oxford, U.K.); mouse TNF-α (Genzyme, Boston, MA); FBS (HyClone, Logan, UT); BALB/c mice (Taconic, Germantown, NY); CBA/CaJ mice (The Jackson Laboratory, Bar Harbor, ME); RNAzol-B (Tel-Test, Friendswood, TX); QuikHyb (Stratagene, La Jolla, CA), TA vector (Invitrogen, San Diego, CA), p755 plasmid (clone 63058, American Type Culture Collection, Manassas, VA); in vitro transcription and RNase protection kits (Ambion, Austin TX); and Alexa488 goat anti-rat IgG and ProLong antifade mounting medium (Molecular Probes, Eugene, OR).

Cell culture

The human THP-1 and mouse WEHI 265.1 monocytic cell lines (TIB 202 and 204, respectively, American Type Culture Collection) were propagated in RPMI and DMEM, respectively, supplemented with 10% FBS and 2×10^{-5} M 2-ME. After PMA differentiation (100 nM), cells were exposed to LPS in RPMI or DMEM (5% FBS) without PMA or 2-ME. The mouse J774A.1 and RAW264.7 monocyte/macrophage lines (TIB 67 and 71, respectively; American Type Culture Collection) were propagated in DMEM with 5% heat-inactivated FBS. For individual experiments, cells were seeded at 1×10^6 cells/10-cm plate in DMEM (5% FBS), grown to ~75% confluence, and treated with LPS. Mouse peritoneal macrophages were cultured as follows. Mice (8- to 12-wk-old females) of the BALB/c or CBA/CaJ strain were sacrificed by cervical dislocation, and unstimulated macrophages were collected by peritoneal lavage with a sterile solution of 0.34 M sucrose. Harvested cells were plated in RPMI (10% FBS) and enriched for macrophages by washing away nonadherent cells after 2 h. The medium was replaced, and cells were treated with vehicle (dH_2O) or LPS. Peritoneal macrophages were elicited from 10- to 12-wk-old C57BL/6J mice by an i.p. injection of 1 ml of 3% thioglycolate broth (Difco, Detroit, MI) 4 days before collection of peritoneal macrophages. The peritoneal macrophages were enriched and cultured as described above.

Metabolic labeling, immunoprecipitation, and immunofluorescent detection of SR-A protein

J774A.1 or RAW264.7 cells were plated at 3.0×10^5 cells/well in six-well plates in phenol red-free DMEM (5% FBS). After the cells had grown to 75% confluence they were treated with LPS for 24 h. To label cellular proteins, we removed the medium and washed the cells in PBS, after which they were incubated in 0.6 ml of leucine-deficient DMEM with 40 μl of L-[U-^{14}C]leucine for 2.5 h at 37°C and chased for 2.5 h by adding 5 ml of DMEM (5% FBS). The SR-A protein was immunoprecipitated with 2F8 mAb as previously described (14), except that protein A-Sepharose was substituted for protein G-Sepharose. For immunofluorescent detection of SR-A, J774.1 cells were grown in chamber slides (Nunc, Naperville, IL) treated with LPS, fixed in 2% paraformaldehyde, blocked with 3% BSA in PBS, and incubated with the 2F8 Ab (2 μg/ml) for 1 h at room temperature. Bound Ab was detected with an Alexa488 fluorophore conjugated to goat anti-rat IgG, nuclei were counterstained with Hoechst 33258, slides were mounted with ProLong antifade mounting medium, and fluorescence was detected with a Nikon EFD-3 scope (Melville, NY).

Northern analysis and DNA probes

Total RNA was isolated with RNAzol B reagent according to the manufacturer's protocol. Isolated RNA (20 μg) was run on 1.0% formaldehyde agarose gels and transferred to nitrocellulose. RNA transfer efficiency and integrity were assessed by ethidium bromide staining. The nitrocellulose membrane was baked at 80°C for 2 h, and hybridization was performed with QuikHyb according to the manufacturer's protocol. RT-PCR was used to amplify and clone a portion of the mouse SR-A gene (15). The sense (5′-TTGGCTTCCCTGGAGGTCGAG-3′) and antisense (5′-ACACAG GAACCAATGTCATTTG-3′) SR-A primers amplified the region between nucleotides 949 and 1233 of the type II isoform, which contains sequences common to both the type I and the type II messages and a region unique to the type II message (16). Amplified RT-PCR products were cloned into the TA vector, and the cloned products were verified by DNA sequencing. For Northern analysis, the SR-A restriction fragment isolated from the TA plasmid was radiolabeled by nick translation with [α-^{32}P]dCTP and the Klenow enzyme to a sp. act. of 2.5×10^6 cpm/ng. For RNase protection assays an antisense riboprobe was generated using the plasmid linearized with *Hin*dIII in an in vitro transcription reaction as recommended by the manufacturer. The mouse c-*fms* probe consisted of the full open reading frame obtained as an *Eco*RI fragment isolated from the p755 plasmid. The mouse TNF-α probe was a gift from H. Xu and G. Hotamisligil (Harvard University School of Public Health, Boston, MA). An oligonucleotide probe for 18S ribosomal RNA (5′-ACGGTATCTGATCGTCTTC GAACC-3′) was used to normalize gene expression (17).

Determination of SR-A message stability, alternative splicing, and transcriptional activity

RAW264.7 cells were cultured in 10-cm plates as described above and were treated for 16 h with 100 ng/ml LPS or an equivalent volume of vehicle (dH_2O). Actinomycin D was then added (10 μg/ml), and total RNA was collected from the cells at various time points after treatment. The RNAs were subjected to Northern analysis, and SR-A transcript levels were quantitated by phosphorimage analysis (Molecular Dynamics, Sunnyvale, CA). A regression analysis (StatView 4.0 software, Abacus Concepts, Berkeley, CA) was used to determine the statistical significance of the message half-life estimates. To determine SR-A type I and type II isoform expression levels, RNase protection assays were performed using the RPA II kit according to the manufacturer's protocol. Nuclear run-on assays were performed to measure the transcriptional activity of the SR-A promoter according to published protocols using purified DNA fragments as probes (18).

Results

LPS differentially regulates SR-A mRNA expression in human monocyte THP-1 and mouse monocyte WEHI-265.1 cells after differentiation to the macrophage phenotype

In cell culture human monocytes differentiate to macrophages and begin to express SR-A (19). During this culture-induced differentiation of human monocytes, exposure to LPS has been shown to inhibit expression of SR-A through a TNF-α-dependent mechanism (10). Similarly, during phorbol ester-induced differentiation, exposure of human THP-1 monocyte-like cells to TNF-α inhibits expression of SR-A (11). In both these experiments exposure to LPS and TNF-α was concomitant with induction of differentiation; thus, these studies do not clarify whether the observed down-regulation of SR-A was confined to the period of differentiation from a monocyte to a macrophage phenotype. To examine this question THP-1 cells were first differentiated by treating them with the phorbol ester PMA for 24 h. The PMA-containing medium was then removed, and the cells were washed with PBS and incubated for another 24 h in the presence or the absence of LPS in a medium that lacked PMA. Under these conditions THP-1 cells maintained a high level of SR-A message expression 24 h after the removal of PMA (Fig. 1*A*). Treatment of these cells with LPS still dramatically suppressed SR-A transcripts (80% reduction in SR-A levels relative to that in PMA-treated cells; Fig. 1*B*). Thus, suppression of SR-A transcripts by LPS in THP-1 cells does not depend on exposure to LPS during the differentiation process, nor does it require the continued presence of PMA.

To test the response of mouse monocyte-like cells to LPS we used the WEHI-265.1 line, which has properties similar to those of human THP-1 monocytoid cells: both types of cells grow in suspension and upon PMA exposure become adherent and take on a stellate macrophage phenotype (20, 21). In contrast to THP-1 cells, exposure of WEHI-265.1 cells to PMA only mildly increased SR-A transcript levels, while subsequent exposure to LPS increased transcripts 6-fold relative to those in untreated cells (Fig. 1, *C* and *D*).

LPS induction of SR-A transcripts is a general response in mouse macrophages

We next studied how SR-A expression responded to LPS in cultured mouse peritoneal macrophages. Unstimulated peritoneal macrophages were collected from wild-type BALB/c or CBA/CaJ mice. After isolated cells had been exposed to LPS or vehicle (dH_2O) for 16 h, total RNA was isolated, and SR-A mRNA was measured relative to 18S ribosomal RNA (Fig. 2*A*). In macrophages from both BALB/c and CBA/CaJ mice, SR-A mRNA increased by ~3-fold (Fig. 2*B*). A population of mouse macrophages rich in infiltrating monocyte-derived macrophages was elicited

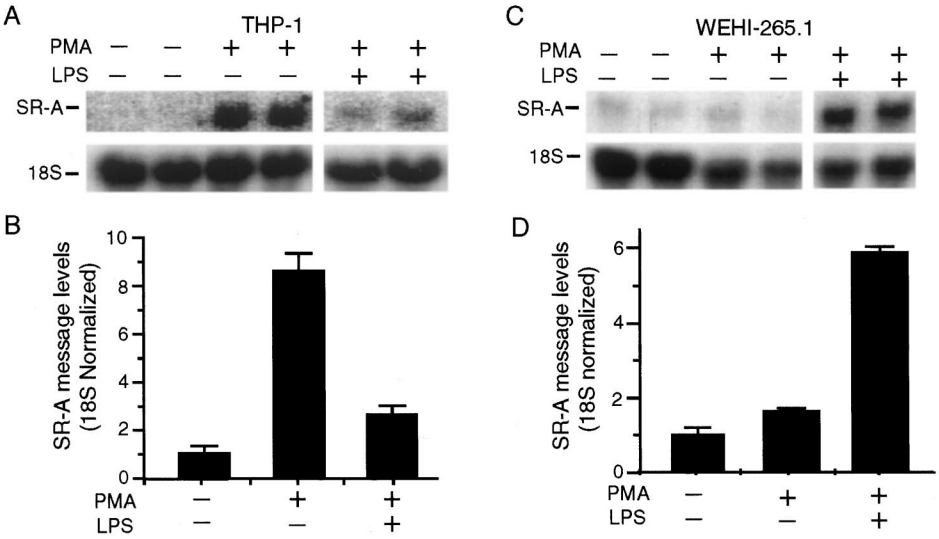

FIGURE 1. LPS down-regulation of SR-A message levels in human THP-1 monocyte/macrophages is not confined to an early differentiation stage and is the opposite of the response of mouse WEHI-265.1 monocyte/macrophages. THP-1 (*A* and *B*) and WEHI-265.1 *(C* and *D)* cells were left undifferentiated (no PMA) or were differentiated with 100 nM PMA for 24 h. The medium was removed, the cells were washed once with PBS, and medium without PMA was added back. The cells were then treated with carrier (dH_2O, 10 μl) or LPS (500 ng/ml, 10 μl) for an additional 24 h. Total RNA (17 μg) was separated on 1% formaldehyde-agarose gels followed by transfer to nitrocellulose membranes. The membrane was then sequentially hybridized with a [^{32}P]DNA probe for SR-A and a [^{32}P]oligonucleotide probe for 18S ribosomal RNA. *A* and *C*, Phosphorimages of the membranes incubated with SR-A and 18S probes. *B* and *D*, Induction of the SR-A message levels normalized to 18S ribosomal RNA (quantitated by phosphorimaging; mean of the two samples ± SE).

from C57BL/6J mice 4 days after peritoneal injection of thioglycolate broth. As with resident peritoneal macrophages, LPS also up-regulated SR-A transcript levels in this population of elicited macrophages (Fig. 2, *C* and *D*).

To further assess whether the up-regulation of SR-A mRNA was a general response of differentiated mouse macrophages we measured the effects of LPS on J774.A1 and RAW264.7 macrophage cells. The J774.A1 and RAW264.7 lines are more differentiated relative to THP-1 and WEHI-265.1 cells because they constitutively adhere to tissue culture plastic and express a variety of macrophage effector functions, including scavenger receptor activity (22). As an indication of their more differentiated phenotype, both lines expressed significant amounts of SR-A transcripts without the need for PMA treatment (Fig. 3*A*, control samples). Exposure to LPS for 24 h caused a significant up-regulation of SR-A mRNA (Fig. 3*A*, LPS-treated samples). Relative to 18S RNA, SR-A transcript levels increased 3- and 7-fold, respectively, in J774.A1 and RAW264.7 cells (Fig. 3*B*). Thus, the LPS-induced increase in mouse macrophage SR-A transcript levels was a general response in both mouse cells representing the monocyte phenotype and

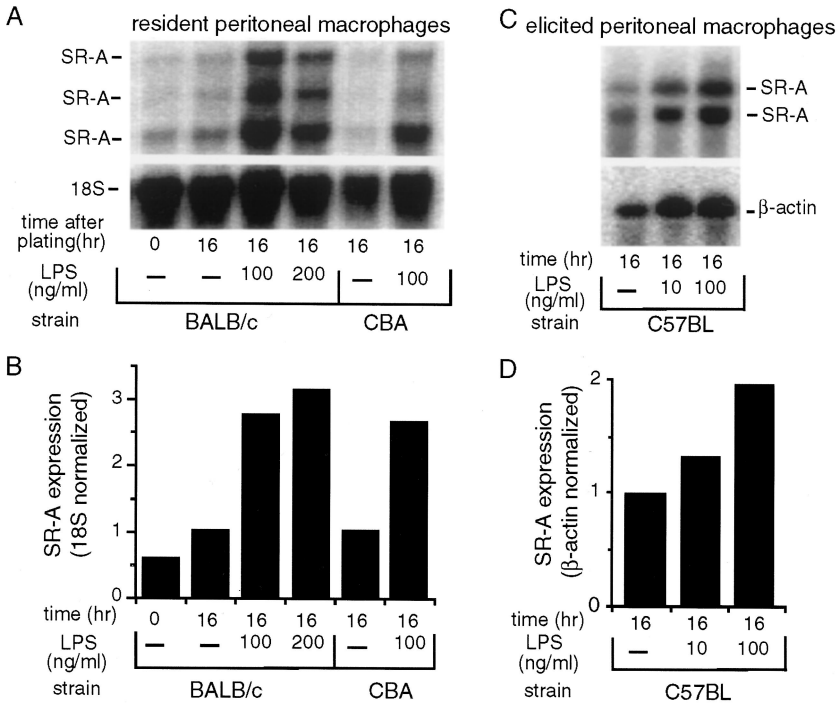

FIGURE 2. Murine peritoneal macrophages up-regulate SR-A message levels in response to LPS. Peritoneal macrophages were collected from unstimulated BALB/c or CBA/CaJ mice (*A*) or from thioglycolate-stimulated C57BL/6J mice (*C*) and enriched by adherence to plastic tissue culture plates. Adherent cells were cultured for 16 h in the presence of the indicated amount of LPS or carrier (dH_2O), and total RNA was isolated and separated on 1% formaldehyde-agarose gels followed by transfer to nitrocellulose membranes. *A* and *C*, Phosphorimages of the membrane sequentially hybridized with a [^{32}P]DNA probe for SR-A and a [^{32}P]oligonucleotide probe for 18S ribosomal RNA or a [^{32}P]DNA probe for β-actin. The 0 h point represents RNA from cells harvested immediately after adhesion to plastic (see *Materials and Methods*). *B* and *C*, Induction of SR-A message levels normalized to 18S ribosomal RNA or β-actin, expressed relative to 16 h controls (quantitated by phosphorimaging).

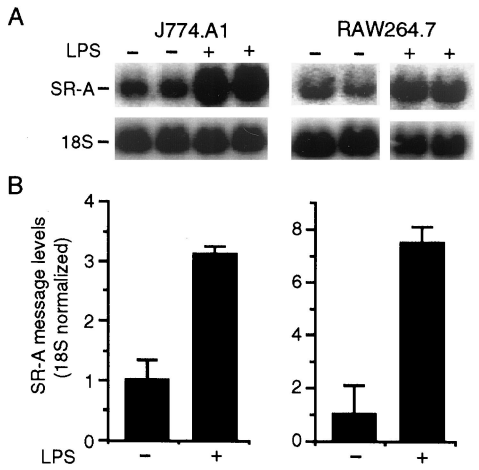

FIGURE 3. LPS induces SR-A message levels in the J774.A1 and RAW264.7 murine macrophage cell lines. J774.A1 and RAW264.7 cells were incubated in the presence of vehicle (dH_2O) or LPS (500 ng/ml) for 24 h. Total RNA (20 μg) was separated on 1% formaldehyde-agarose gels followed by transfer to nitrocellulose membranes. *A*, Phosphorimage of the membranes sequentially hybridized with a [^{32}P]DNA probe for SR-A and a [^{32}P]oligonucleotide probe for 18S ribosomal RNA. *B*, Induction of SR-A message levels normalized to 18S ribosomal RNA (quantitated by phosphorimaging; mean of the two samples ± SE).

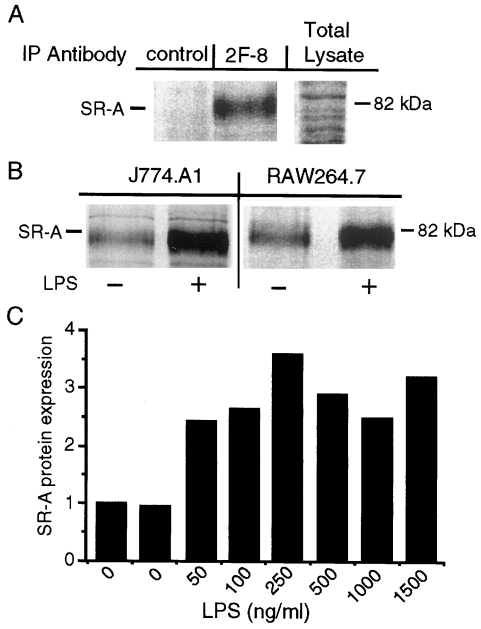

FIGURE 4. LPS induces SR-A protein expression in J774.A1 and RAW264.7 cells. *A*, Specific immunoprecipitation of SR-A from metabolically labeled J774.A1 cells. J774.A1 cells were metabolically labeled with [^{14}C]leucine for 2.5 h in leucine-deficient medium and then chased for 2.5 h in complete medium. Washed cells were lysed, and the lysate (equal amounts of cell associated [^{14}C]leucine) was incubated with an irrelevant mAb (control) or with 2F8, an anti-SR-A I and II mAb (14). Immunoprecipitated proteins were separated by reducing SDS-PAGE and detected by phosphorimaging. The 2F8 Ab specifically precipitated a protein of the appropriate M_r (80 kDa) for SR-A, while the irrelevant control mAb did not. *B*, Induction of SR-A protein levels by LPS treatment of J774.A1 or RAW264.7 cells. Cells were treated with carrier (dH_2O) or LPS (100 ng/ml) for 24 h. SR-A protein was labeled, immunoprecipitated, and analyzed as described in *A*. *C*, Dose response of SR-A protein induction by LPS. J774.A1 cells were treated with increasing concentrations of LPS for 24 h. Cells were labeled with [^{14}C]leucine, and SR-A protein was immunoprecipitated and analyzed as described in *A*. Immunoprecipitated SR-A was quantitated by phosphorimaging. The graph shows the amount of SR-A protein in cells treated with LPS normalized to that in control cells.

those representing a more differentiated tissue macrophage phenotype.

LPS induces SR-A protein in mouse J774.A1 and RAW264.7 cells

To test whether induction of SR-A message levels by LPS in J774.A1 and RAW264.7 cells leads to increased SR-A protein expression, we performed pulse-chase labeling experiments with [^{14}C]leucine. ^{14}C-labeled SR-A protein (80 kDa) was immunoprecipitated from cell lysates using the 2F8 anti-mouse SR-A mAb (Fig. 4*A*). The specificity of the 80-kDa band was demonstrated by its absence in the control precipitate (Fig. 4*A*). SR-A protein levels increased significantly in J774.A1 and RAW264.7 cells exposed to LPS for 24 h compared with those in control cells (treated with dH_2O; Fig. 4*B*). In J774.A1 cells SR-A protein levels were measured relative to total cell-associated [^{14}C]leucine. Increased expression of SR-A protein was detectable after LPS treatment at the lowest dose tested (50 ng/ml; Fig. 4*C*), and the response reached saturation at approximately a 3-fold increase in protein levels.

We further characterized the J774.A1 response to LPS by determining whether increased cell surface expression of SR-A could be detected using immunofluorescence. Compared with control cells, LPS-treated cells showed a marked increase in staining of the cell membrane and cytoplasmic extensions (Fig. 5, compare *A* and *B*). No significant cell immunofluorescence was seen in experiments in which the anti-SR-A Ab was omitted (Fig. 5*C*). These results indicate that up-regulation of SR-A protein demonstrated by metabolic labeling and immunoprecipitation was correlated with increased cell surface expression.

Dose response and time course of SR-A message induction by LPS in RAW264.7 cells

The pulse-chase studies indicated that 50 ng/ml LPS was nearly saturating for induction of SR-A protein. In RAW264.7 cells treated with LPS at 10–500 ng/ml, the increase in SR-A mRNA saturated at 10 ng/ml (data not shown). An additional dose-response experiment using doses from 0.1 to 10 ng/ml showed significant induction of SR-A message by LPS at a concentration as low as 1 ng/ml (Fig. 6*A*). This increase in SR-A message was detectable as early as 8 h after LPS exposure (100 ng/ml), peaked at 16 h, and was still significantly elevated vs control levels at 48 h (Fig. 6*B*). The induction of SR-A transcripts appears to be an intermediate response to LPS, since we observed morphological changes in RAW264.7 cells as early as 3 h after LPS treatment (data not shown).

Induction of SR-A transcripts by LPS requires de novo protein synthesis

The delayed kinetics of SR-A transcript induction (Fig. 6*B*) suggested that de novo protein translation may be necessary for the response to LPS. To test this possibility, we exposed RAW264.7 cells to LPS for 12 h in the presence or the absence of cycloheximide, an inhibitor of eukaryotic protein synthesis. The 4-fold increase in SR-A transcripts induced by LPS was completely blocked by the concomitant addition of cycloheximide (Fig. 7, *A* and *B*). Thus, translation of new proteins is required for LPS induction of SR-A transcripts in RAW264.7 cells.

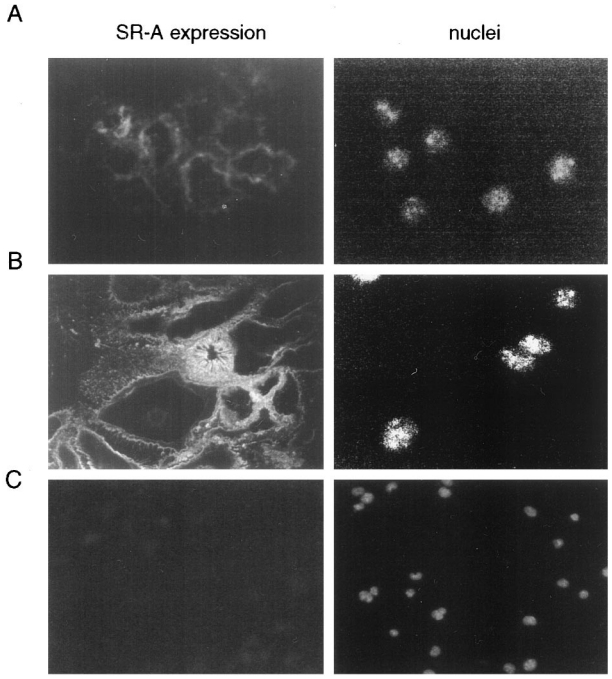

FIGURE 5. LPS treatment increases cell surface SR-A protein expression on J774.A1 cells. J774.A1 cells grown in eight-well chamber slides treated with carrier (dH_2O) or LPS (100 ng/ml) for 16 h were fixed in 2% paraformaldehyde and incubated with (*A* and *B*) or without (*C*) anti-SR-A mAb. Bound Ab was detected with a goat anti-rat fluorescent Ab. Nuclei of the cells were counterstained with Hoechst 33258. *A*, Control cells; *B* and *C*, LPS-treated cells. SR-A fluorescence is shown in the panels on the *left*, while the nuclear stain of the cells is shown on the *right*. Original magnification: *A* and *B*, ×400; *C*, ×200.

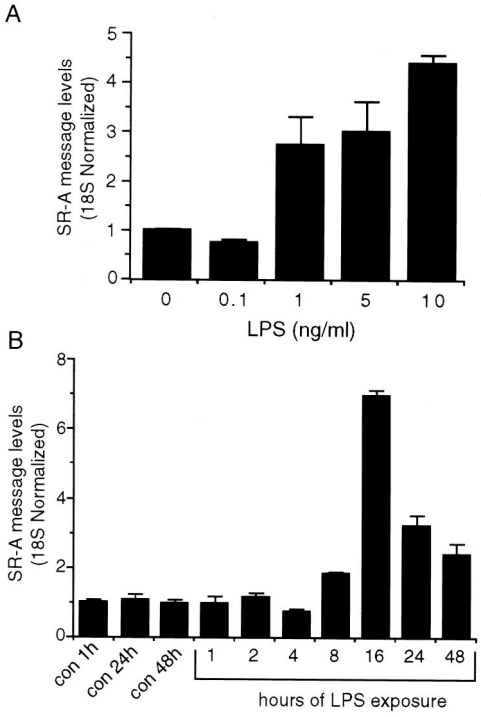

FIGURE 6. LPS increases RAW264.7 SR-A message levels in a dose- and time-dependent fashion. *A*, Dose response of SR-A transcript induction to LPS exposure. RAW264.7 cell were treated with increasing concentrations of LPS from 0.1–10 ng/ml as indicated. Total RNA was isolated and analyzed for SR-A expression as described in Fig. 1. *B*, Time course of SR-A transcript induction in response to LPS exposure. RAW264.7 cells were treated with LPS (100 ng/ml) for increasing periods of time as indicated. Control cells were treated with an equivalent volume of dH_2O and harvested at the indicated times thereafter. Total RNA was isolated and analyzed for SR-A expression as described in Fig. 1. All samples in the experiments shown in *A* and *B* were performed in duplicate. The SR-A message levels were normalized to the 18S ribosomal RNA levels (quantitated by phosphorimaging). The graphs show the mean ± SE and are representative of two such experiments.

Induction of SR-A mRNA by LPS does not involve alternative splicing or transcriptional activation

We used RNase protection assays to further define whether the mechanism of SR-A transcript induction by LPS involved splicing effects, because SR-A type I and II isoforms are alternative transcripts derived from one open reading frame (16). We found that in unstimulated cells the majority of transcripts coded for the type II isoform (79%; Fig. 8*A*), and that LPS did not selectively alter splicing, because SR-AI transcripts were increased to the same extent as SR-AII transcripts (8.0- vs 7.7-fold, respectively).

Next we determined whether LPS modulated the transcriptional activity of the murine SR-A locus using the nuclear run-on technique (18). SR-A transcriptional activity found in nuclei from cells treated with LPS for 16 h did not differ significantly compared with the activity in nuclei of control treated cells (LPS, 104 ± 11% of control; $p = 0.59$; $n = 4$; Fig. 8*B*). The steady state SR-A transcripts were induced 8 ± 0.7-fold ($n = 3$) during these experiments as demonstrated in Fig. 8*C*. We also tested for alteration of transcriptional activity at earlier time points during LPS exposure (3, 6, 9, and 12 h) and again found no evidence for induction of SR-A transcriptional activity (Fig. 8*D*).

LPS induction mildly increases SR-A message half-life and occurs in the presence of actinomycin D

To test whether LPS altered SR-A message stability, we used actinomycin D to block transcription in RAW264.7 cells that had been treated with 100 ng/ml LPS for 16 h. Total RNA was collected, and SR-A mRNA, TNF-α mRNA, and 18S ribosomal RNA were measured by Northern blotting (Fig. 9*A*). The rapid decay of TNF-α transcripts indicates that transcription was effectively blocked by actinomycin D in both control and LPS-treated cells. By regression analysis the rate of decay of SR-A mRNA (Fig. 9*B)* was only mildly increased in the LPS-treated cells relative to that in control cells (8.6 ± 2.4 vs 6.8 ± 1.8 h, respectively). In these studies we noted that SR-A transcript levels did not immediately begin to decay after the addition of actinomycin D, suggesting that LPS induction may occur in the presence of actinomycin D when transcription is inhibited. To explore this further we treated RAW264.7 cells with actinomycin D after only 6 h of LPS exposure and analyzed SR-A and TNF-α transcripts (Fig. 9*C*). Again, TNF-α transcripts rapidly decayed after actinomycin D treatment, indicating that transcription had been effectively blocked. In contrast, SR-A mRNA levels in LPS-treated cells continued to increase for up to 4–4.5 h after treatment with actinomycin D (Fig. 9*C*). To further examine the significance of the increase in SR-A transcripts in the presence of actinomycin D we searched for additional genes whose transcripts were induced by LPS. We found that FcγRII transcripts were induced by LPS in RAW264.7 cells with kinetics similar to those of SR-A transcripts, but to a greater extent (maximal induction at 16 h of 25- vs 8-fold; compare Fig. 6*B* and Fig. 9*D*). In contrast to its effects on SR-A transcripts (Fig. 9*C*), actinomycin blocked induction of FcγRII transcripts (in the same experiments; Fig. 9*E*).

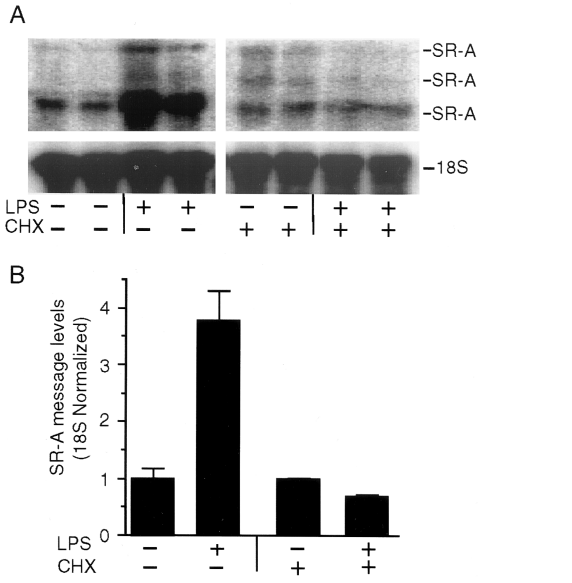

FIGURE 7. Cycloheximide treatment of RAW264.7 macrophages blocks the LPS-induced increase in SR-A transcripts. RAW264.7 cells were treated with either cycloheximide (10 μg/ml) or vehicle (dH_2O) in the presence or the absence of LPS (100 ng/ml). Cell were harvested after 12 h of treatment, and total RNA was isolated. *A*, Northern analysis of isolated total RNA (15 μg) for SR-A message levels and 18S ribosomal RNA. *B*, Induction of SR-A message levels normalized to the 18S ribosomal RNA levels (quantitated by phosphorimaging). The graph shows the mean of the duplicate samples ± SE, with the results representative of two such experiments.

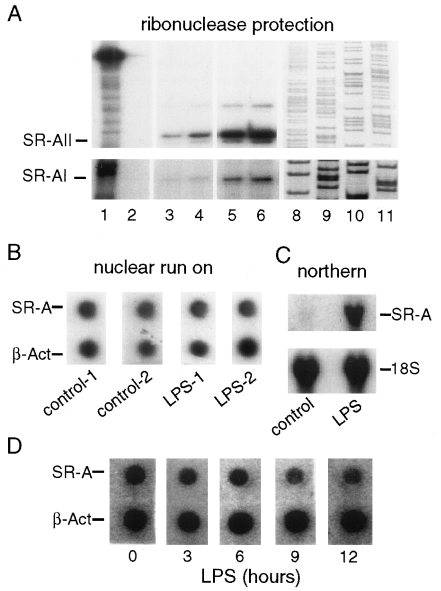

FIGURE 8. Induction of SR-A mRNA by LPS does not involve alternative splicing or transcriptional activation. RAW264.7 cells were exposed to vehicle (dH_2O), or LPS (100 ng/ml) for 16 h. *A*, Total RNA was isolated, and the levels of SR-AI and II transcripts were determined by RNase protection assay (*lane 1*, undigested probe; *lane 2*, probe digested with 20 μg of yeast transfer RNA; *lanes 3* and *4*, probe digested with 20 and 40 μg of RNA (controls); *lanes 5* and *6*, probe digested with 20 and 40 μg of RNA (LPS); *lanes 7–10*, DNA markers). *B* and *D*, Nuclear run-on assays were performed on nuclei isolated from RAW264.7 cells treated with LPS as described in *A* or for the indicated times. Shown is a representative experiment with duplicate samples probed for nascent SR-A and β-actin transcripts. Results are representative of three independent experiments. *C*, Northern analysis of total SR-A transcripts from cells cultured and treated in parallel to those used in *B*.

TNF-α exposure does not recapitulate the induction of SR-A mRNA in RAW264.7 cells exposed to LPS

It has been reported that the down-regulation of SR-A expression by LPS in human THP-1 cells occurs through an autocrine/paracrine mechanism driven by TNF-α secretion (11). We examined whether TNF-α treatment of mouse RAW264.7 cells recapitulated the LPS-induced increase in SR-A transcripts. Cells were treated with mouse TNF-α or LPS for 24 h, and total RNA was isolated and analyzed for SR-A transcripts (Fig. 10*A*, *top panel*). As expected, LPS exposure resulted in a 4.5-fold increase in SR-A transcripts relative to 18S RNA levels (Fig. 10*B*). TNF-α exposure, however, resulted in a modest decrease in SR-A expression. Because expression of the M-CSF receptor gene (c-*fms*) responds to both TNF-α and LPS, we probed for c-*fms* transcripts (Fig. 10*A*, *middle panel*). The level of c-*fms* transcripts relative to 18S RNA decreased by about 40% after TNF-α exposure and by >80% after LPS exposure (Fig. 10*B*). Thus, although TNF-α was able to modulate c-*fms* transcripts, it was not able to recapitulate the LPS induction of SR-A transcripts in RAW264.7 mouse macrophages.

Discussion

The capacity of SR-A to mediate the internalization and degradation of LPS has implicated the receptor in the clearance of LPS by macrophages (7). The role of SR-A in protecting mice from LPS-induced shock has been demonstrated recently using SR-A knockout animals in a model of endotoxic shock (8). Studies of human monocyte-derived macrophages appeared to contradict these ideas, because LPS strongly down-regulated scavenger activity and SR-A expression in these cells (9, 10). A potential explanation for this paradox was that the suppression of SR-A transcripts by LPS was confined to an early period of the differentiation process. Phorbol ester-induced differentiation of human THP-1 monocytes indicated that this was not the case, because after conversion to the macrophage phenotype, as evidenced by increased adherence and robust expression of SR-A, LPS still significantly decreased SR-A expression.

In contrast, peritoneal macrophages cultured from two mouse strains increased SR-A transcript levels by 3-fold when exposed to an identical serotype of LPS. This up-regulation of SR-A appears to be a general response of differentiated mouse macrophages, because J774.A1 and RAW 264.7 macrophage cells also responded to LPS by increasing steady state levels of SR-A transcripts. Because these cells may all represent resident tissue macrophages, we used the WEHI 265.1 line as a surrogate for mouse monocyte/macrophages (20, 21). After PMA exposure to induce a macrophage phenotype, these cells up-regulated SR-A expression in response to LPS. In addition, thioglycolate-elicited cells, which are enriched in infiltrating monocyte/macrophages, also up-regulate SR-A in response to LPS. The fact that these cells only up-regulated SR-A expression 2-fold in response to LPS is consistent with the observation that a variety of inflammatory stimuli, such as thioglycolate broth, elicit SR-A expression in mice (23). Thus, for resident mouse macrophages, mouse macrophages rich in infiltrating monocyte/macrophages, and three mouse macrophage cell lines, LPS induces a different pattern of SR-A expression relative to that reported for human monocyte/macrophages.

In the J774.A1 and RAW264.7 macrophage lines, LPS treatment increased protein expression by 3-fold, and this increase was associated with increased cell surface expression. These results argue for the functional significance of LPS induction, because

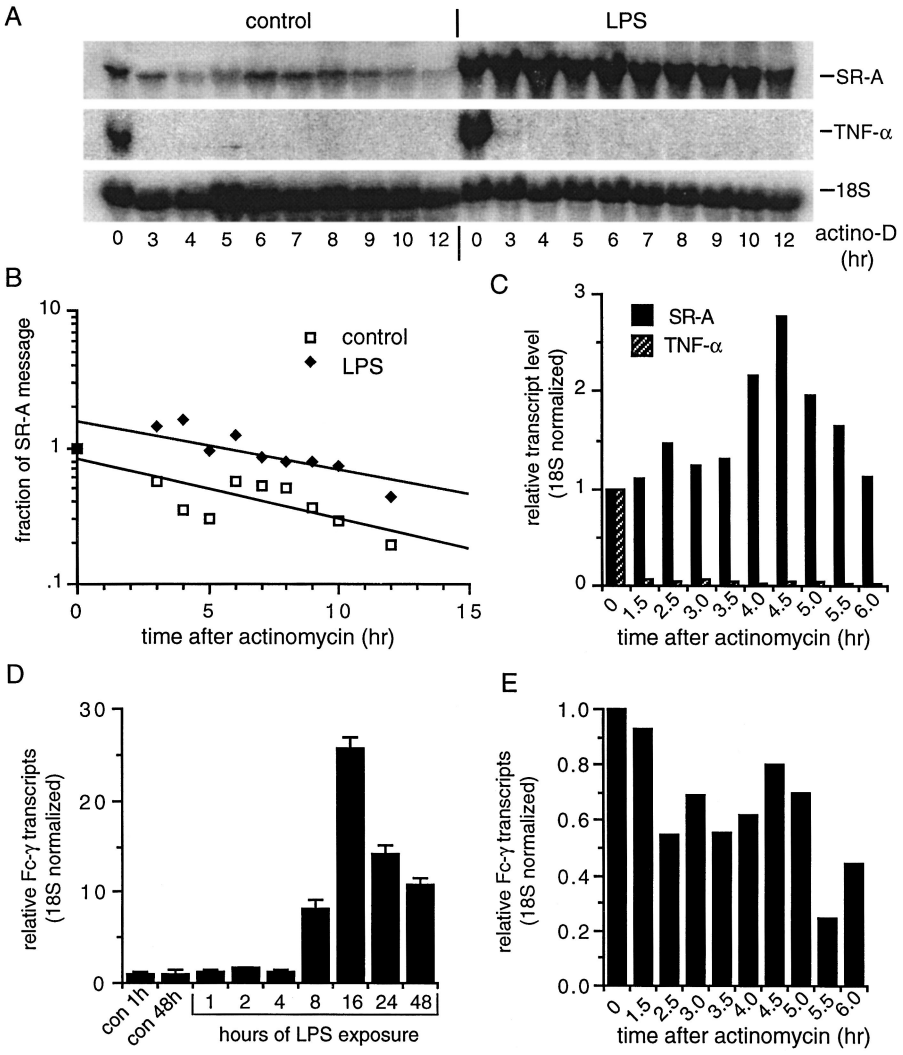

FIGURE 9. Effects of LPS on SR-A message half-life in actinomycin D-treated cells. RAW264.7 cells were treated with either LPS (100 ng/ml) or carrier (dH_2O) for 16 h. The cells were then treated with actinomycin D (10 μg/ml) for the indicated times, and total RNA was isolated. *A*, Northern analysis of isolated total RNA for SR-A and TNF-α transcripts and 18S ribosomal RNA. *B*, The SR-A message levels (quantitated by phosphorimaging) were normalized for total amount of RNA loaded (20 μg, except controls at 5–12 h, for which 40 μg was loaded to obtain an adequate signal), expressed relative to levels at time zero, and graphed on a semilog scale. The mRNA half-lives were determined by least squares regression analysis of the data. *C*, RAW264.7 cells were treated with 100 ng/ml LPS for 6 h and then exposed to actinomycin D (10 μg/ml) for the indicated times, and total RNA was isolated. SR-A and TNF-α transcripts and 18S ribosomal RNA levels were assessed by Northern analysis (not shown). SR-A and TNF-α transcripts are expressed relative to 18S normalized transcript levels immediately before actinomycin D addition. *D*, RAW264.7 cells were treated with 100 ng/ml LPS or vehicle for the indicated times, and levels of FcγRII transcripts were determined by Northern analysis. *E*. Expression of FcγRII transcripts levels in the RNA samples shown in *C*.

overexpression of SR-A increases the internalization and degradation of LPS, presumably through trafficking to the lysosome (7), without activating cellular signaling (24). The increase in SR-A transcripts occurred at a low level of LPS (1 ng/ml), with the response saturating at 10 ng/ml. These levels are physiologically relevant, because the 50% lethal dose in 129 mice is 34 $\pm$ 6.6 ng/ml, while that in SR-A knockout animals is significantly lower (4.7 $\pm$ 1.5 ng/ml) (8). Interestingly, human monocyte/macrophages display a similar dose response to LPS, because 1 ng/ml LPS down-regulates SR-A transcripts, with maximal suppression seen at 10 ng/ml (9).

The induction of SR-A transcripts by LPS was seen by 8 h, peaked at 16 h, and persisted for 48 h. These intermediate kinetics suggested that the LPS effect on SR-A expression may require the translation of novel protein factors. In support of this idea, we found that the protein synthesis inhibitor cycloheximide blocked the induction of SR-A transcripts by LPS. Studies at different times after stimulation showed that LPS did not enhance the transcriptional activity of the SR-A locus. To analyze LPS effects on transcript stability, we used standard conditions of actinomycin D treatment and found that LPS induction mildly increased mature SR-A message half-life from 6.8 $\pm$ 1.8 to 8.6 $\pm$ 2.4 h. This slight lengthening of half-life induced by LPS did not adequately explain the increase in SR-A transcripts. However, the effects of LPS on SR-A transcript levels in the presence of actinomycin D were complex. Control cells appeared more sensitive then LPS-treated cells to actinomycin D toxicity. We do not believe that this difference affected the half-life of SR-A transcripts such that it was an overestimate. If actinomycin D had artificially stabilized transcripts in a generalized manner, such an effect should have also been operative on TNF-α transcripts. This was not the case, as TNF-α transcripts rapidly decayed by 3 h (Fig. 9*A*). These results indicated that the increase in mature LPS RNA transcripts was not simply explained by either transcriptional activation or stabilization of mature mRNA.

When we tested the effect of actinomycin D at 6 h post-LPS treatment, a time when the levels of processed transcripts were actively increasing, we found that mature SR-A transcripts continued to accumulate for up to 4.5 h after the addition of actinomycin D. These results suggests three potential interpretations: 1) that actinomycin D failed to fully inhibit transcription; 2) that SR-A transcription was differentially sensitive to actinomycin D; or 3) that although actinomycin D did inhibit transcription, the primary SR-A transcripts or intermediates present at the time of addition of actinomycin only became detectable later as a result of post-transcriptional mRNA processing. The first interpretation seems unlikely, because, under the same conditions, gene transcription was inhibited for both TNF-α and FcγRII (which is induced with similar kinetics, but to a greater magnitude than the SR-A gene). Although we cannot completely rule out that the

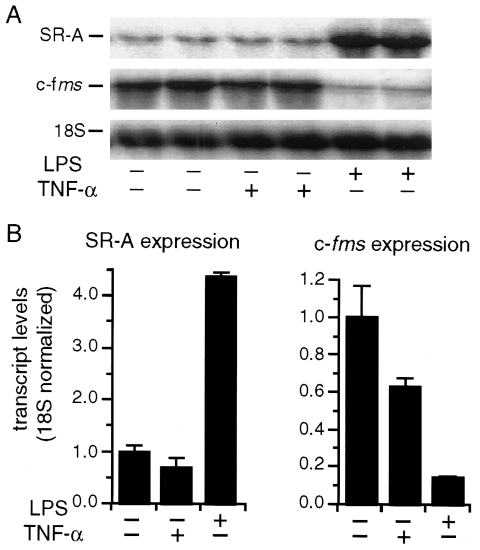

FIGURE 10. TNF-α does not up-regulate SR-A transcripts in RAW264.7 cells. RAW264.7 cells were exposed to vehicle (dH_2O), TNF-α (200 U/ml), or LPS (100 ng/ml) for 24 h. *A*, Total RNA was isolated, and expression of SR-A transcripts, c-*fms* transcripts, and 18S ribosomal RNA was determined by Northern analysis as before. The panels show SR-A transcripts (*top*), c-*fms* transcripts (*middle*), and 18S ribosomal RNA (*bottom*). *B*, Relative levels of SR-A and c-*fms* transcripts normalized to 18S ribosomal RNA levels quantitated by phosphorimaging (mean of the two samples ± SE).

SR-A locus was differentially sensitive to transcriptional inhibition, such a possibility appears unlikely, because our nuclear run-on studies did not detect increased SR-A transcript initiation in response to LPS.

Overall, our results are most consistent with the idea that LPS induced SR-A transcripts by a post-transcriptional process that affected message maturation or export. It is known that the induction of lysozyme transcripts by LPS involves regulated sequential splicing of primary transcripts (25). This type of regulated mRNA splicing and export is known to occur in response to signaling pathways involving growth factors, nutritional status, and environmental stress (25–28). However, we were unable to uncover direct evidence that LPS induced an alteration in the post-transcriptional processing of SR-A transcripts. This may be because our method of isolating RNA did not recover all of the nuclear RNA, particularly the very large (60-kb) primary SR-A transcripts. Still, we found that LPS did not alter the ratio of type I or II messages, as determined by RNase protection assays. In fact, the pattern of SR-A transcript expression visualized by Northern blotting appeared to vary among macrophages of the various mouse stains and among the cell lines studied. These transcripts represent splice variants of a single gene (16), with the larger m.w. type II transcripts thought to arise from alternative termination and polyadenylation sites in the 3′-untranslated portion of the SR-A gene (16). LPS did not appear to differentially affect the use of alternative termination or polyadenylation sites, because in the BALB/c-derived peritoneal macrophages all three transcript sizes were induced to a similar extent. Further studies are needed to determine whether the LPS induction of SR-A transcripts involves regulated sequential splicing of primary transcripts (25) or alters SR-A message export.

These studies highlight the differences between SR-A expression in mouse and human cells. In contrast to the 6-h half life of SR-A transcripts in RAW264.7 cells, the half-life of SR-A message in differentiated human THP-1 cells is much longer (40 h) and declines precipitously (10 h) when THP-1 cells are exposed to TNF-α (11). A further divergence in the regulation of mouse SR-A was found, because TNF-α did not induce the expression of SR-A in RAW264.7 cells. Because the biological activity of TNF-α was confirmed by the down-regulation of c-*fms* transcripts, this experiment indicates that TNF-α signaling alone is not sufficient to recapitulate the LPS-induced increase in SR-A expression in RAW264.7 cells.

The finding that mouse macrophages up-regulate SR-A expression when exposed to LPS is consistent with the in vivo observation that SR-A protects against LPS toxicity. Injection of LPS into the hippocampus of wild-type BALB/c mice was correlated with increased SR-A expression on infiltrating macrophages and microglia (23). Furthermore, macrophages from wild-type mice primed with Calmette-Guérin bacillus expressed scavenger receptor activity, and when SR-A knockout mice were challenged with a systemic dose of LPS, they were more susceptible to endotoxic shock (8). Additional studies are needed to test whether in mice the LPS-induced up-regulation of SR-A expression increases resistance to endotoxemia (29).

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