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# Regulation of Toll-Like Receptor 4 Expression in the Lung Following Hemorrhagic Shock and Lipopolysaccharide<sup>1</sup>

Jie Fan,\* Andras Kapus,\* Philip A. Marsden,<sup>†</sup> Yue Hua Li,\* George Oreopoulos,\* John C. Marshall,\* Stefan Frantz,<sup>‡</sup> Ralph A. Kelly,<sup>‡</sup> Ruslan Medzhitov,<sup>§</sup> and Ori D. Rotstein<sup>2\*</sup>

The Toll-like receptor 4 (TLR4) has recently been shown to function as the major upstream sensor for LPS. In this study, a rodent model of lung injury following resuscitated hemorrhagic shock was used to examine the regulation of TLR4 gene and protein expression *in vivo* and *in vitro*. Intratracheal LPS alone induced a rapid reduction in whole lung TLR4 mRNA, an effect which is also observed in recovered alveolar macrophages. This effect appeared to be due to a lowering of TLR4 mRNA stability by ~69%. By contrast, while shock/resuscitation alone had no effect on TLR4 mRNA levels, it markedly altered the response to LPS. Specifically, antecedent shock prevented the LPS-induced reduction in TLR4 mRNA levels. This reversal was explained by the ability of prior resuscitated shock both to prevent the destabilization of TLR4 mRNA by LPS and also to augment LPS-stimulated TLR4 gene transcription compared with LPS alone. Oxidant stress related to shock/resuscitation appeared to contribute to the regulation of TLR4 mRNA, because supplementation of the resuscitation fluid with the antioxidant *N*-acetylcysteine reversed the ability of shock/resuscitation to preserve TLR4 mRNA levels following LPS. TLR4 protein levels in whole lung mirrored the changes seen for TLR4 mRNA. Considered in aggregate, these data suggest that levels of *tlr4* expression are controlled both transcriptionally as well as posttranscriptionally through altered mRNA stability and that antecedent shock/resuscitation, a form of global ischemia/reperfusion, might influence regulation of this gene. *The Journal of Immunology*, 2002, 168: 5252–5259.

The development of the acute respiratory distress syndrome in individuals following major trauma contributes significantly to morbidity and mortality in this patient population (1, 2). Resuscitated hemorrhagic shock is believed to promote the development of lung injury by priming the immune system for an exaggerated inflammatory response to a second, often trivial, stimulus, the so-called “two hit hypothesis” (3). Using a rodent model, we previously demonstrated that a period of sustained shock followed by resuscitation leads to augmented lung neutrophil sequestration and lung injury in response to a small dose of intratracheal LPS (4). This effect appeared to be mediated by the increased LPS-stimulated release from alveolar macrophages of the chemokine, cytokine-induced neutrophil chemoattractant (CINC),<sup>3</sup> the rat ortholog of IL-8. In those studies, alveolar macrophages from animals undergoing resuscitated shock showed earlier and increased expression of CINC mRNA and protein, as well as enhanced nuclear translocation of NF- $\kappa$ B in response to LPS stimulation. Considered together, these findings suggested that resuscitated hemorrhagic shock augments LPS-induced lung injury

by increasing the responsiveness of the cellular signaling pathways to LPS challenge.

Recent studies have provided further insights into the signaling pathways whereby LPS causes dissociation of I- $\kappa$ B from the cytoplasmic I- $\kappa$ B/NF- $\kappa$ B complex and leads to nuclear translocation of NF- $\kappa$ B. The Toll-like receptor 4 (TLR4), a member of the Toll-like receptor family, has been shown to serve as the main upstream sensor for LPS effect *in vitro* and *in vivo*. Cells derived from *tlr4*-deficient mice or mice with a spontaneous mutant TLR4 are known to be resistant to LPS (5, 6), while unresponsive HEK 293 cells are rendered responsive to LPS following transfection with TLR4 cDNA (7). Two other cell surface molecules, CD14 and MD-2, also appear necessary to optimize LPS signaling. CD14, in its soluble form or as a GPI-anchored surface protein, may act as a signaling bridge to promote surface recognition of LPS by the cell (7–9). MD-2 is physically associated with TLR4 on the cell surface and appears to both augment the responsiveness of the cell to LPS and also regulate activation of specific intracellular signaling pathways following LPS engagement (10–12). Recent studies suggest that TLR4, CD14, and MD-2 may serve as a tripartite LPS receptor complex with LPS binding to each of the component molecules (13).

Expression of *tlr4* has been demonstrated in several tissues and is particularly pronounced among myelomonocytic cells (5, 14). However, *tlr4* regulation in these cells remains largely unexplored. Muzio et al. (14) reported that LPS increased levels of TLR4 mRNA in human peripheral blood monocytes in an actinomycin D-dependent fashion, suggesting transcriptional regulation. By contrast, Poltorak et al. (5) demonstrated that TLR4 mRNA was constitutively present in RAW 264.7 cells and LPS rapidly and transiently suppressed TLR4 mRNA levels. Similarly, Nomura et al. (15) observed that LPS treatment of mouse peritoneal macrophages lowered both TLR4 mRNA levels and surface TLR4 expression. These studies did not examine whether alterations in TLR4 mRNA levels were due to altered transcription, mRNA

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<sup>3</sup> Abbreviations used in this paper: CINC, cytokine-induced neutrophil chemoattractant; MAP, mean arterial pressure; NAC, *N*-acetylcysteine; TLR4, Toll-like receptor 4; RL, Ringer's lactate; SAL, saline alone; BAL, bronchoalveolar lavage.

stability, or both. The present studies were performed to examine the mechanisms regulating *tlr4* gene expression in vivo and also to evaluate whether resuscitated hemorrhagic shock, a known predisposing event for the development of lung inflammation in man, might influence its regulation. In this paper, we show that *tlr4* is regulated both transcriptionally and posttranscriptionally following exposure to LPS and that antecedent resuscitated hemorrhagic shock modulates the nature of the LPS response.

## Materials and Methods

### *Animal model of resuscitated hemorrhagic shock and lung injury*

Male Sprague-Dawley rats (300–350 g; Charles River Breeding Laboratories, St. Constant, Quebec, Canada) were anesthetized with 80 mg/kg ketamine and 8 mg/kg xylazine administered i.p. The right carotid artery was cannulated with a 22-gauge angiocath (BD Biosciences, Franklin Lakes, NJ) for monitoring of mean arterial pressure (MAP), blood sampling, and resuscitation. Hemorrhagic shock was initiated by blood withdrawal and reduction of the MAP to 40 mmHg within 15 min, as previously described. This blood pressure was maintained by further blood withdrawal if the MAP was >45 mmHg, and by infusion of 0.5 ml of Ringer's lactate (RL) if the MAP was <35 mmHg. Blood was collected into 0.1 ml citrate per milliliter of blood to prevent clotting. After a hypotensive period of 60 min, animals were resuscitated by transfusion of the shed blood and RL in a volume equal to that of shed blood, over a period of 2 h. In some studies, animals received *N*-acetylcysteine (NAC) (0.5 g/kg) via the artery before the infusion of RL. The catheter was then removed, the carotid artery was ligated, and the cervical incision was closed. Sham animals underwent the same surgical procedures, but hemorrhage was not induced. NAC delivery occurred in sham animals at an equivalent time to that received in shock animals (4).

Altered gene expression in the lung after shock/resuscitation was studied using two protocols, one in vivo and the other ex vivo (16). For in vivo studies, animals underwent a tracheotomy using a 14-gauge catheter 1 h after the end of resuscitation (or sham), and then received either LPS (30  $\mu$ g/kg *E. coli* O111:B4 in 200  $\mu$ l saline) or saline alone (SAL) intratracheally followed by 20 mechanically ventilated breaths using a rodent ventilator. Therefore, the experimental protocol was described as having animals in one of four groups: sham/SAL, shock/SAL, sham/LPS, and shock/LPS. Using this protocol, we previously showed that animals subjected to shock/LPS exhibited increased lung permeability and neutrophil counts compared with all other groups, while sham/LPS group had a small increase in bronchoalveolar lavage (BAL) neutrophil count but no change in permeability (4). At various time points after LPS (or SAL) administration ( $t = 0$ ), whole lung was recovered for examination by Western or Northern blot analysis. Ex vivo studies used alveolar macrophages retrieved by BAL following shock/resuscitation (or sham). At this time point, there was no difference between groups with respect to the total cell count in the BAL fluid or the absolute number of alveolar macrophages recovered. Macrophages obtained from shock/resuscitated or sham rats were then incubated for various times at 37°C in 5% CO<sub>2</sub> either alone or in the presence of 0.1  $\mu$ g/ml LPS. At the end of the incubation period, cells were pelleted by centrifugation at 300  $\times g$  for 10 min. Cells were then processed as indicated for analysis by flow cytometry, Western blot, or Northern blot.

### *Bronchoalveolar lavage*

BAL was performed via the intratracheal catheter using cold PBS (8 mM disodium phosphate, 2 mM potassium phosphate, 0.14 M sodium chloride, and 0.01 M potassium chloride (pH 7.4), with 0.1 mM EDTA). BAL fluid was centrifuged at 300  $\times g$  for 10 min to pellet cells. The cell pellet was then suspended on NIM.2 neutrophil isolation medium (Cardinal Associates, Santa Fe, NM), and centrifuged at 750  $\times g$ , 20°C, for 45 min for macrophage isolation. The isolated macrophages were washed and resuspended in DMEM containing 10% FCS at a concentration of 1  $\times 10^6$  cells/ml medium in polypropylene tissue culture tubes. Cell viability was >95%, as assessed by trypan blue exclusion, and consisted of a cell population of >95% macrophages, as assessed by Wright-Giemsa staining.

### *Northern blot analysis*

Total RNA from lungs or alveolar macrophages was obtained using the guanidium-isothiocyanate method (17). Briefly, lungs or macrophages were harvested and immediately frozen in liquid nitrogen. Tissue or cells were then thawed and homogenized in 4 M guanidine-isothiocyanate con-

taining 25 mM sodium citrate, 0.5% sarcosyl, and 100 mM 2-ME. RNA was denatured, electrophoresed through a 1.2% formaldehyde-agarose gel, and transferred to nylon membrane. Hybridization was conducted using a [<sup>32</sup>P]dCTP-labeled TLR4 cDNA (kindly provided by Dr. S. Frantz, Brigham and Women's Hospital, Boston, MA; see Ref. 18), TNF- $\alpha$  cDNA (American Type Culture Collection, Manassas, VA), and a [<sup>32</sup>P]ATP-end-labeled 30-base oligonucleotide probe for CINC, which is complementary to nucleotides 134–164 of CINC cDNA (19). Blots were then washed under conditions of high stringency and specific mRNA bands were detected by autoradiography in the presence of intensifying screens, as previously reported. Blots were stripped and reprobated for GAPDH to control for loading. Expression of mRNA was quantitated using a PhosphoImager and accompanying ImageQuant software (Molecular Dynamics, Sunnyvale, CA) and was normalized to the GAPDH signal.

### *Nuclear run-on analysis*

LPS treated and untreated alveolar macrophages obtained from the ex vivo experiments were washed with sterile 10 mM PBS and lysed in situ with chilled lysis buffer (10 mM Tris-HCl (pH 7.9), 0.15 M NaCl, 1 mM EDTA, and 0.6% (v/v) Nonidet P-40) for 10 min on ice. Cell lysates were centrifuged for 5 min at 500  $\times g$  at 4°C, and the nuclear pellet was resuspended in chilled nuclear buffer containing 0.3 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 100 mM Tris-HCl (pH 7.9), 4 mM MgCl<sub>2</sub>, 4 mM MnCl<sub>2</sub>, 0.2 M NaCl, 0.4 mM EDTA, 0.1 mM PMSF, and 40% (v/v) glycerol. Cold transcription buffer (0.3 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 100 mM Tris-HCl (pH 7.9), 4 mM MnCl<sub>2</sub>, 0.2 M NaCl, 0.4 mM EDTA, and 0.1 mM PMSF) containing 0.2 mM DTT, 40 U RNasin, 0.2 mM ATP, CTP, and GTP, and 150  $\mu$ Ci [ $\alpha$ -<sup>32</sup>P]UTP (3000 Ci/mmol) was added to the nuclear suspension and incubated for 30 min at 28°C. A total of 20 U of RNase-free DNase I and 125  $\mu$ g tRNA were added and incubated for 10 min at 37°C, followed by digestion with proteinase K at a final concentration of 300  $\mu$ g/ml in buffer (10 mM Tris-Cl (pH 7.9), 10 mM EDTA, 0.5% SDS) for 30 min at 42°C. Nuclear transcripts were then extracted as described above and resuspended at 2  $\times 10^6$  cpm/ml in Northern hybridization buffer. Equal amounts (1  $\mu$ g) of gel-purified cDNA were denatured by boiling in 0.4 M NaOH and 10 mM EDTA, neutralized with equal volumes of 2 M ammonium acetate (pH 7), and slot-blotted onto nitrocellulose filters. Hybridization was performed for 48 h at 42°C in Northern hybridization buffer as detailed above (20). Hybridized filters were washed and subjected to autoradiography and quantitation as described above.

### *Actinomycin D chase experiments*

To assess the effect of LPS and hemorrhage/resuscitation on the half-life of TLR4 mRNA transcripts, alveolar macrophages from shock/resuscitated and sham animals were treated with or without LPS for 0.5 h before addition of 10  $\mu$ g/ml actinomycin D. Total cellular RNA was extracted at 0, 15, 30, 60, 120, 180, and 240 min after the addition of actinomycin D. Blots were reprobated with a GAPDH cDNA probe to ensure equal loading. TLR4 mRNA transcript levels were normalized for GAPDH mRNA using ImageQuant, and mRNA decay rates were determined according to the following formula:  $t_{1/2} = 0.693/k$  where  $k = \ln(N_0/N_t)/t$ , where  $N_0$  represents the density at  $t = 0$  and  $N_t$  represents the density at time  $t$ . Results are the mean from three independent experiments.

### *Western blot analysis*

The lung tissue homogenate was separated on an 8% SDS-PAGE under reducing conditions (21). Equivalent loading of the gel was determined by quantitation of protein as well as by Coomassie staining of the gel. Separated proteins were electroblotted onto polyvinylidene difluoride membrane and blocked for 1 h at room temperature with TBS containing 1% BSA. The membranes were then incubated with a 1/2500 dilution of antiserum against human TLR4 (Dr. R. Medzhitov, Brigham and Women's Hospital) at room temperature for 1 h. Ag-Ab complexes were identified with goat anti-rabbit IgG tagged with HRP (Sigma-Aldrich, St. Louis, MO) and exposed to the ECL detection system (Amersham, Arlington Heights, IL) according to the manufacturer's instructions.

### *Statistics*

The data are presented as mean  $\pm$  SE of  $n$  determinations as indicated in the figures. Data were analyzed by one-way analysis of variance; post hoc testing was performed using the Bonferroni modification of the  $t$  test. When individual studies are demonstrated, these are representative of at least three independent studies.



## Results

### *Effect of shock/resuscitation on TLR4 expression in vivo*

Fig. 1A shows levels of TLR4 mRNA expression under the various experimental conditions. The  $t = 0$  time point represents tissues analyzed 1 h after end resuscitation before intratracheal LPS challenge. As shown, whole lungs from sham animals demonstrated constitutive expression of TLR4 mRNA, and shock/resuscitation had no effect on these levels. Over the 6-h experimental period, the levels of TLR4 mRNA in both sham/SAL and shock-alone animals remained essentially stable compared with  $t = 0$ . By contrast, sham/LPS animals showed a reduction in TLR4 mRNA by 2 h after LPS, with near complete disappearance of mRNA expression by 4 h. The response to LPS in the lungs of animals exposed to prior resuscitated shock (shock/LPS) differed from that seen for LPS-alone animals. The rapid early reduction noted for LPS alone was less pronounced, with the levels tending to increase toward control beginning at 4 h. Levels of the housekeeping gene GAPDH remained constant throughout and did not differ between groups.

Several cell types in the lung may be potential sources of TLR4 expression, including alveolar macrophages, exudative neutrophils, and endothelial cells (14, 18, 22). Because we previously showed that enhanced release of chemokines and inflammatory cytokines by alveolar macrophages contributed to augmented lung injury (4, 16), we recovered these cells after resuscitated shock (or sham) to evaluate their TLR4 gene expression according to the *ex vivo* protocol. This approach also permitted evaluation of TLR4 in macrophages, free of contamination by other cells. As noted in *Materials and Methods*, neither total cell counts nor the percentage of macrophages in the BAL differed between shock and sham animals. The Northern blot analysis of TLR4 mRNA shown in Fig. 1B illustrates that the pattern observed using isolated alveolar macrophages was similar to that seen for whole lung. Specifically, while cells from sham/SAL and shock/SAL exhibited steady levels of TLR4 mRNA, LPS treatment of cells recovered from sham animals resulted in rapid reduction in levels of expression, with a tendency toward late restoration of control levels. By contrast, TLR4 mRNA levels in alveolar macrophages recovered from shock/resuscitated animals remain essentially unchanged following LPS treatment over the course of the experimental period. A dose response examining the effect of LPS alone on alveolar macrophages demonstrates that 0.01  $\mu\text{g/ml}$  LPS causes a slight reduction in levels of TLR4 mRNA, with progressive diminution as the dose was increased up to 1  $\mu\text{g/ml}$  (Fig. 1Ca). We also studied later time points following LPS treatment. As shown in Fig. 1Cb, levels of TLR4 mRNA in cells cultured *in vitro* for 18 h after being recovered from shock/resuscitated animals did not differ from sham animals, while LPS treatment for 18 h caused marked reduction in TLR4 mRNA levels whether cells were recovered from sham or shock/resuscitated animals.

The rapid reduction in mRNA levels in cells treated with LPS was not observed when macrophages were treated with other inflammatory stimuli *in vitro*. As shown in Fig. 1D, neither TNF- $\alpha$  (Fig. 1D, upper panels) nor IL-1 $\beta$  (Fig. 1D, lower panels), given at concentrations shown to up-regulate macrophage CD11b expression *in vitro* (data not shown), altered TLR4 mRNA expression in alveolar macrophages. Considered together, these findings suggest that shock/resuscitation per se has little effect on TLR4 expression in lung macrophages but markedly changes the responsiveness of these cells to LPS. In addition, the events occurring in LPS (or vehicle)-stimulated alveolar macrophages following their recovery from sham or shock/resuscitated animals appear to accurately reflect *in vivo* events following intratracheal LPS (or vehi-

cle) and thus constitute a relevant model for studying regulation of TLR4 in these cells.

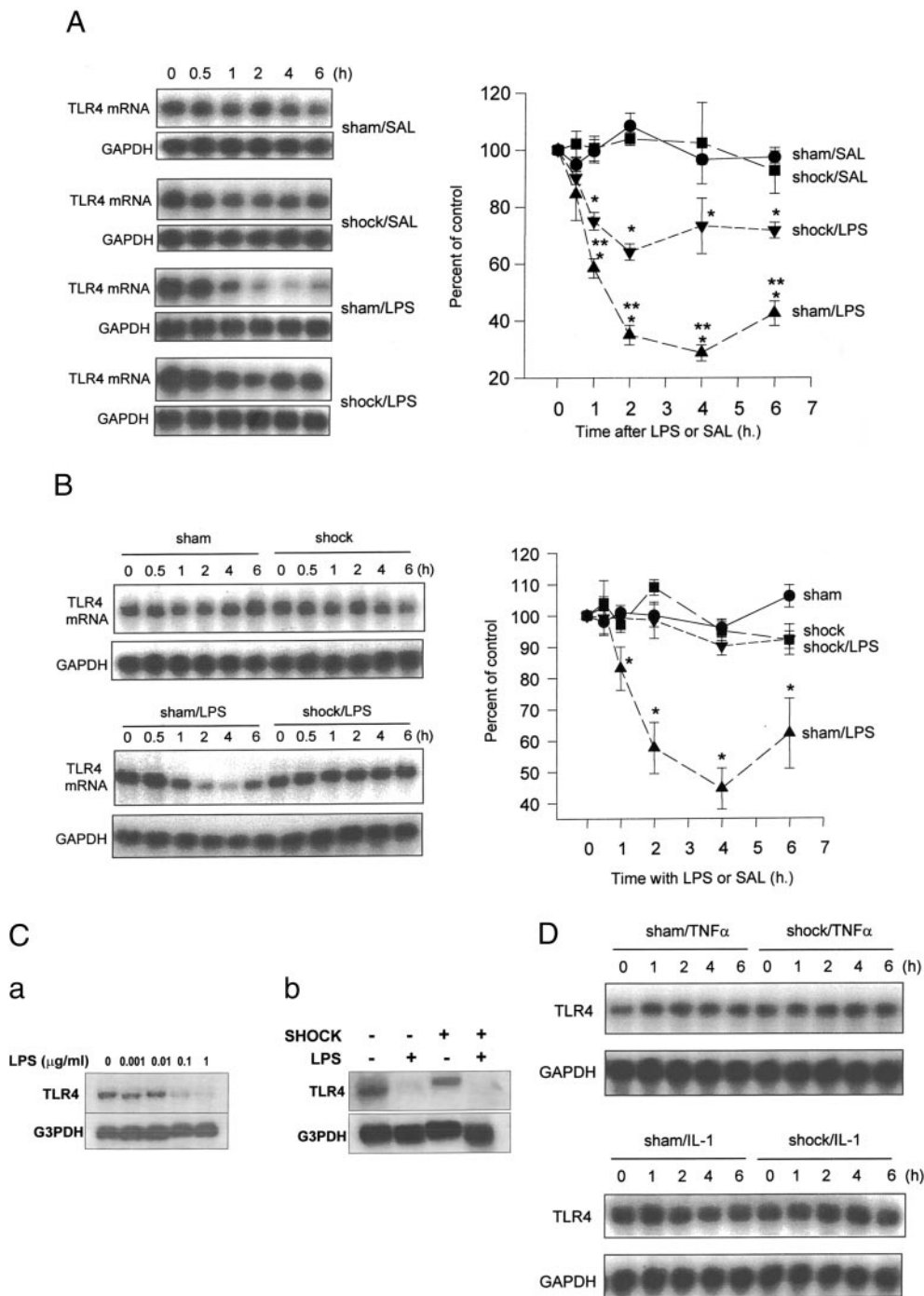
### *Regulation of TLR4 mRNA expression*

Steady state mRNA levels represent a balance between message stability and rate of gene transcription. We addressed both possibilities to investigate the mechanism of altered mRNA expression. The rapid reduction in TLR4 mRNA levels following LPS treatment in cells from sham animals suggested the possibility that message stability might be altered. Consistent with this hypothesis, LPS treatment of cells derived from sham animals shortened the half-life of TLR4 mRNA compared with untreated cells by  $\sim 69\%$ , representing a reduction from  $168 \pm 32$  to  $53 \pm 5$  min (mean  $\pm$  SD;  $n = 3$ –5 per group;  $p < 0.001$ ) (Fig. 2). This effect of LPS on TLR4 mRNA half-life was not observed in cells recovered from animals following resuscitated shock. Shock alone caused a small increase in TLR4 mRNA half-life ( $192 \pm 51$  min;  $n = 3$ ), while shock/LPS cells exhibited a TLR4 mRNA half-life of  $139 \pm 19$  min ( $n = 5$ ; not significant compared with shock alone), representing an  $\sim 28\%$  reduction compared with shock alone. Shock/LPS did not differ from sham. These findings suggest that reduced TLR4 mRNA stability following LPS in sham animals may account for the rapid lowering in levels of TLR4 mRNA. In addition, the relative lack of destabilization of TLR4 message in shock/resuscitated animals may contribute to the differential effects of LPS on TLR4 mRNA levels between sham and shock animals.

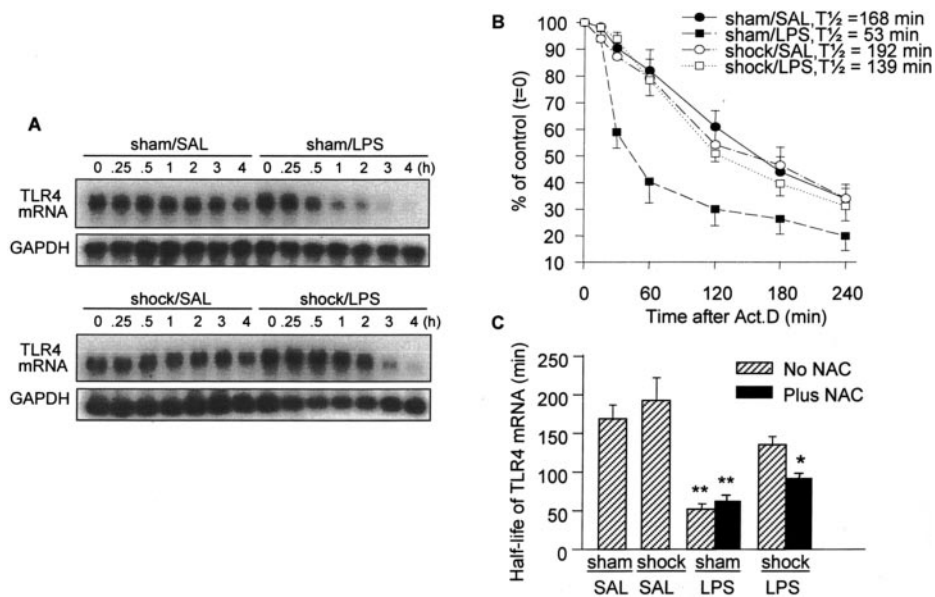
We also considered the possibility that shock/resuscitation regulated TLR4 mRNA through an effect on transcription. Transcriptional run-on assays using macrophages derived from sham and shock animals are illustrated in Fig. 3A. In the absence of treatment, cells recovered in the BAL fluid exhibited constitutive transcription of TLR4, a finding consistent with the basal levels of TLR4 mRNA evident *in vivo* and *in vitro*. The transcription rate was similar in shock/resuscitated animals. In cells recovered from sham animals and then exposed to LPS, there was an  $\sim 1.4$ -fold increase in the *tlr4* transcription rate, while LPS treatment of cells from shock/resuscitated animals resulted in an  $\sim 9$ -fold rise in transcription. Densitometry derived from three independent studies is shown in Fig. 3B. Considered together with the mRNA stability studies, these findings suggest that the preservation of TLR4 mRNA levels in shock/LPS animals is related to a combination of enhanced mRNA stability plus increased mRNA transcription. In addition, while sham/LPS caused a small increase in the *tlr4* transcription rate, this effect was outweighed by the marked reduction in the mRNA stability in terms of determining overall TLR4 mRNA levels.

### *Role of oxidant stress in regulating TLR4 expression in alveolar macrophages*

Increased circulating xanthine oxidase released from the gastrointestinal tract occurs following hemorrhagic shock (23) and has been shown to contribute to subsequent lung gene expression (24). We have previously shown that supplementation of the resuscitation fluid with NAC prevented lung injury and lessened the LPS-induced CINC and TNF mRNA and protein following shock/resuscitation, suggesting a role for oxidant stress in the priming process (4). To examine whether generated oxidants might influence the regulation of *tlr4* gene expression following shock/LPS, we evaluated TLR4 expression either in whole lung derived from NAC-resuscitated animals or in macrophages recovered after shock/resuscitation by BAL (Fig. 4). In the absence of NAC, TLR4 mRNA expression was again maintained in shock/LPS groups compared with LPS alone, whether examined in whole lung (Fig.



**FIGURE 1.** Alterations in TLR4 mRNA expression in whole lung and alveolar macrophages. *A*, TLR4 mRNA expression in whole lung from sham and shock animals at  $t = 0-6$  h following intratracheal LPS or SAL (*left panel*). Scanning densitometry values of Northern blot for TLR4 mRNA are normalized by densitometry of corresponding GAPDH mRNA bands (*right panel*) and expressed as mean  $\pm$  SEM ( $n = 4$  rats per group; \*,  $p < 0.01$  vs sham/SAL; \*\*,  $p < 0.01$  vs shock/LPS). *B*, TLR4 mRNA expression in alveolar macrophages. Alveolar macrophages were recovered and enriched from BAL fluid after resuscitated shock or sham treatment and then treated in vitro with or without LPS ( $0.1 \mu\text{g/ml}$ ) for the time indicated. Total RNA was extracted from the cells, and Northern blot analysis was performed. The *left panel* shows a representative result of three independent studies and the *right panel* shows the densitometric analysis for TLR4 mRNA after normalization with GAPDH. Results are expressed as mean  $\pm$  SEM ( $n = 3$ ; \*,  $p < 0.01$  vs other groups). *C*, Dose response and time course of TLR4 mRNA expression in alveolar macrophages. *Ca*, Alveolar macrophages were recovered and enriched from BAL fluid after sham treatment, incubated in vitro with varying concentrations of LPS for 4 h, and then subjected to Northern blot analysis. GAPDH was used to ensure comparable loading of lanes. The blot is representative of three independent studies. *Cb*, Alveolar macrophages were recovered and enriched from BAL fluid after resuscitated shock or sham treatment and then treated in vitro with or without LPS ( $0.1 \mu\text{g/ml}$ ) for 18 h. Total RNA was extracted from the cells, and Northern blot analysis was performed. GAPDH was used to ensure comparable loading of lanes. The blot is representative of two independent studies. *D*, Effect of TNF- $\alpha$  and IL-1 $\beta$  on TLR4 mRNA expression in alveolar macrophages. Alveolar macrophages were recovered from BAL fluid after resuscitated shock or sham treatment and then were incubated in vitro with or without either TNF- $\alpha$  ( $20 \text{ ng/ml}$ ) or IL-1 $\beta$  ( $200 \text{ U/ml}$ ) for 0–6 h. Each blot is representative of three independent studies.



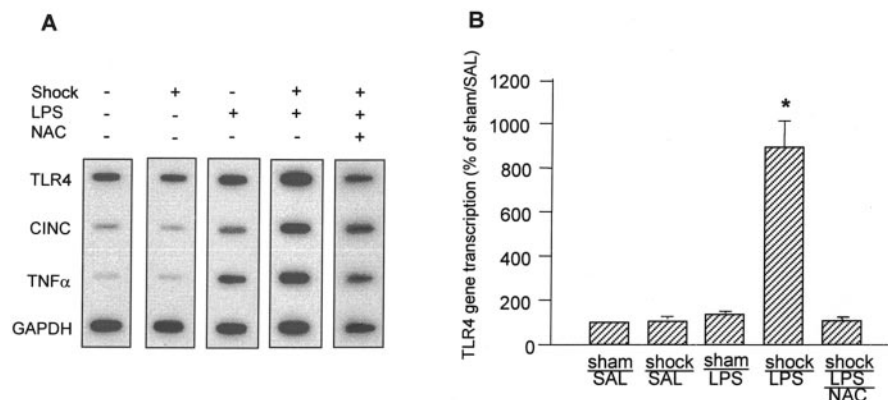
**FIGURE 2.** Effect of shock and LPS on TLR4 mRNA stability. *A*, Representative Northern blot analysis of the disappearance of TLR4 mRNA in alveolar macrophages after transcriptional blockade at  $t = 0$  with actinomycin D ( $10 \mu\text{g}/\text{ml}$ ). Alveolar macrophages were collected from the BAL fluid of sham or resuscitated shock animals, and then were incubated in the presence or absence of LPS ( $0.1 \mu\text{g}/\text{ml}$ ) for 0.5 h followed by actinomycin D treatment. Total cellular RNA was extracted at various time points after addition of actinomycin D. Blots were rehybridized with GAPDH probe for loading differences between lanes. The blot is representative of three to five independent studies for each condition. *B*, TLR4 mRNA levels were determined by densitometry and then normalized by GAPDH. The half-life for TLR4 mRNA was then calculated from three (sham/SAL and shock/SAL group) or five (sham/LPS and shock/LPS group) independent experiments as indicated in *Materials and Methods* (mean  $\pm$  SEM; \*,  $p < 0.01$  vs other groups). *C*, Effect of NAC on TLR4 mRNA half-life. NAC ( $0.5 \text{ g}/\text{kg}$ , intra-arterially) was administered to animals in sham or shock groups and then macrophages were recovered and stimulated with LPS in vitro. Transcriptional arrest was accomplished using actinomycin D as noted above and TLR4 mRNA half-life was determined as in *Materials and Methods*. These studies were performed in parallel with those in *B* and are expressed as a histogram for comparison to these results. Data represent mean  $\pm$  SEM. \*,  $p < 0.01$  vs shock/LPS without NAC; \*\*,  $p < 0.01$  vs all other No NAC groups;  $n = 3\text{--}5$  studies per group.

4A) or in recovered macrophages (Fig. 4B). However, NAC prevented the effect of shock/LPS on preservation of TLR4 mRNA levels. Specifically, animals resuscitated with NAC demonstrated markedly reduced levels of TLR4 mRNA following LPS treatment compared with shock/LPS alone. This effect appeared to be mainly due to an effect on *tlr4* transcription. As demonstrated in Fig. 3A, NAC lowered the *tlr4* transcription rate to that observed in untreated cells. Altered mRNA stability may have also contributed to the effect of NAC on TLR4 mRNA levels, because NAC supplementation of the resuscitation fluid reduced TLR4 mRNA stability by  $\sim 32\%$  (Fig. 2C). NAC did not influence the LPS-induced re-

duction in TLR4 mRNA stability (Fig. 2C). These data suggest that oxidant stress resulting from shock/resuscitation contributes to the altered TLR4 gene regulation in response to LPS treatment.

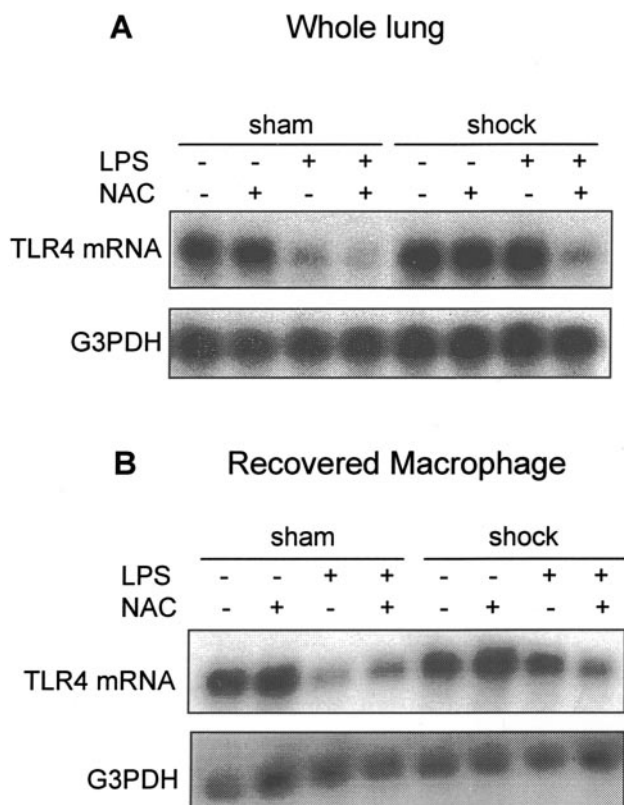
#### Effect of shock/LPS on TLR4 protein expression in vivo

Having demonstrated that shock modulated TLR4 mRNA levels in response to LPS exposure, we evaluated its effect on TLR4 protein levels. Whole lung was recovered from animals at varying times after LPS (or SAL) administration and evaluated for TLR4 protein by Western blot analysis. As shown in Fig. 5, whole lung TLR4 remained stable over the 4-h period after intratracheal SAL in both



**FIGURE 3.** Effect of shock and LPS on the rate of alveolar macrophage gene transcription. *A*, Gene transcription rate in alveolar macrophages. Alveolar macrophages were harvested by BAL from rats following shock/resuscitation or sham treatment, and then were incubated with or without LPS ( $0.1 \mu\text{g}/\text{ml}$ ) for 2 h. *Far right panel*, NAC ( $0.5 \text{ g}/\text{kg}$ , intra-arterially) was added to the resuscitation fluid following shock. Isolated cell nuclei were allowed to generate nuclear run-on products in the presence of [ $^{32}\text{P}$ ]UTP and then hybridized to linear, immobilized cDNA probes as indicated. One representative of three independent studies is shown. *B*, The TLR4 gene transcription rate was normalized using GAPDH and expressed as mean  $\pm$  SEM (\*,  $p < 0.01$  vs other groups;  $n = 3$ ).



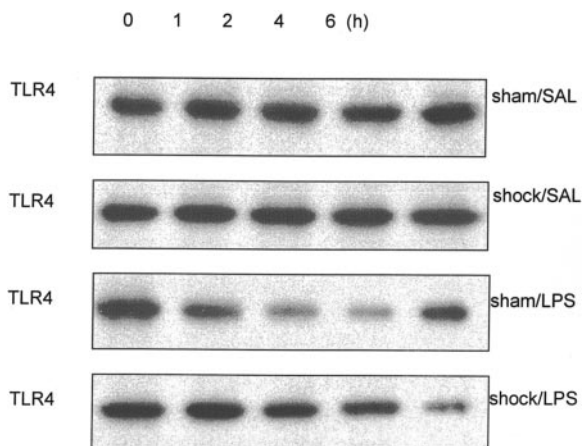


**FIGURE 4.** Northern blot showing the effect of NAC (0.5 g/kg) on expression of TLR4 mRNA extracted from the whole lung tissue (A) or from alveolar macrophages (B) following resuscitated shock (or sham) treatment at  $t = 4$  h after intratracheal LPS instillation. A representative of three independent experiments is shown.

sham and shock animals. By contrast, whole lung TLR4 levels rapidly diminished in animals following LPS treatment. This reduction was markedly attenuated in LPS-treated animals following antecedent resuscitated shock.

## Discussion

Recent investigation has established TLR4 as a major receptor for LPS-induced responses in myeloid cells as well as in other cell



**FIGURE 5.** Effect of resuscitated shock on LPS-induced changes in whole lung TLR4 protein. Western blot analysis with anti-TLR4 Ab was performed on lung tissue sampled from shocked or sham rats at  $t = 0-6$  h after LPS or SAL intratracheally. One representative of three independent experiments is shown.

types. The present study examines *tlr4* gene expression under pathophysiological conditions in vivo and evaluates cellular and molecular mechanisms contributing to its regulation. The major findings are the following: 1) There is significant basal expression of TLR4 mRNA transcripts in the lung. Intratracheal LPS causes a rapid reduction in levels of TLR4 mRNA in the lung, an effect that is recapitulated in alveolar macrophages. The reduction appears to be due primarily to destabilization of the mRNA with a shortening of the  $t_{1/2}$  by  $\sim 69\%$ . 2) Shock/resuscitation modulates the response to LPS. In both whole lung and alveolar macrophages, antecedent-resuscitated shock prevents the down-regulation of TLR4 in response to LPS so that TLR4 mRNA levels are maintained near control levels. Two mechanisms appear to contribute to this preservation. First, the reduction in mRNA stability observed with LPS alone is reversed when cells are subjected to antecedent-resuscitated shock. Second, shock/resuscitation primes for increased transcription of *tlr4* in response to LPS. 3) Oxidant stress induced by hemorrhage/resuscitation contributes to the maintenance of TLR4 mRNA levels. The use of the antioxidant NAC in the resuscitation fluid prevents the priming for increased TLR4 mRNA transcription and partially reverses the prolongation of TLR4 mRNA half-life observed following shock/resuscitation. 4) Whole lung expression of TLR4 protein mirrors changes in mRNA levels. Together, these studies provide new insight into the regulation of *tlr4* gene and protein expression by LPS in vivo and its modulation by antecedent-resuscitated shock, a form of global ischemia/reperfusion.

In this rodent model, macrophages exhibited significant basal levels of *tlr4* gene expression and exposure to LPS exerted a net effect of decreasing steady state mRNA and protein levels, both in vitro and in vivo. Other investigators have reported the constitutive expression of TLR4 mRNA in cells of myeloid lineage and its stimulus-specific rapid reduction in mRNA levels (5, 15, 25). However, the present studies are the first to evaluate the mechanism underlying this effect of LPS. Specifically, while LPS was shown to cause both new gene transcription and destabilization of TLR4 mRNA transcripts, it appears that destabilization of TLR4 mRNA transcripts predominated during the early time course of the studies, thereby lowering steady state TLR4 mRNA levels. Other investigators have reported different basal expression of TLR4 and altered responsiveness of cells to LPS. For example, Frantz et al. (18) reported that neither cardiac myocytes nor coronary microvascular endothelial cells exhibited significant constitutive expression of TLR4 mRNA, and that LPS treatment increased TLR4 expression in both. Differences between studies may be related to the cell type studied as well as the species of origin. These may differ in basal transcription rates of the *tlr4* gene as well as the relative effects of LPS on destabilization of the TLR4 mRNA transcript and stimulation of *tlr4* gene transcription. These studies also underscore the fact that, in addition to new gene transcription, the metabolic fate of mRNA transcripts following cellular activation may influence steady state level of mRNA transcripts. That cell stimulation can induce destabilization of mRNA transcripts has been reported for inducible NO synthase, the  $\beta$ -adrenergic receptor, and the estrogen- $\alpha$  receptor, among others (26-28). It remains to be determined whether LPS also destabilizes the mRNA transcript in man, as it does in the rat.

The maintenance of TLR4 mRNA levels in shock/resuscitated animals following LPS treatment appears to be related to two mechanisms. First, antecedent shock markedly increased LPS-induced *tlr4* gene transcription compared with the response to LPS in sham animals. Second, LPS-induced TLR4 mRNA destabilization was reduced in cells exposed to prior shock/resuscitation compared with sham animals. The latter of these two mechanisms

seems to be the predominant one, at least within the first 2 h after LPS treatment. Oxidants appear to participate in the regulation of *tlr4* gene expression. NAC supplementation during resuscitation markedly reduced levels of TLR4 mRNA detected in shock/LPS animals at  $t = 4$  h following LPS administration. This appears to be primarily mediated via an effect on the *tlr4* transcription rate, which is diminished to basal levels by NAC administration, although a lesser effect on TLR4 mRNA stability was observed. The mechanism of the NAC-sensitive augmentation in *tlr4* gene transcription remains to be defined. The most plausible explanation is that oxidants enhance signaling through the TLR4-NF- $\kappa$ B cascade and thus promote augmented LPS-induced gene transcription. Evidence supporting an oxidant effect on this signaling pathway is derived from two observations made in our prior reports. First, NAC addition during resuscitation prevented the augmented NF- $\kappa$ B translocation in macrophages following LPS treatment. Second, NAC supplementation reversed the enhanced LPS-induced gene transcription of the NF- $\kappa$ B-dependent genes CINC and TNF in animals exposed to antecedent shock/resuscitation. One alternate possibility is that NAC precluded up-regulation of an NF- $\kappa$ B-dependent gene whose product stimulates TLR4 transcription through an NF- $\kappa$ B-independent pathway. For example, enhanced generation of an NF- $\kappa$ B-dependent gene product such as TNF- $\alpha$  may synergize with the effects of LPS on TLR4 transcription and lead to an augmented rise in TLR4 in shock/LPS animals. A recent report by Frantz et al. (29) demonstrated that surface TLR2 was required for oxidant-induced nuclear translocation of NF- $\kappa$ B in neonatal rat cardiac myocytes. These authors suggested that factor(s) released from oxidant-treated cells might activate TLR2. As noted above, further elucidation of the role of NF- $\kappa$ B in *tlr4* gene regulation is necessary to support these hypotheses. Ischemia/reperfusion is known to activate stress-activated protein kinase and ultimately promote AP-1-dependent reporter activity in rat myocytes (30). Thus, the AP-1 consensus binding sequence in the promoter of the *tlr4* gene may also represent one potential target for modulating TLR4 transcription in cells recovered from shock/resuscitated animals and then exposed to LPS.

Prior studies by Nomura et al. (15) demonstrated that the rapid reduction in TLR4 surface expression following LPS treatment correlated with reduced cytokine release in response to a second exposure to LPS. These investigators suggested that this down-regulation of surface receptors might contribute to the development of LPS tolerance. Based on the present studies, one might speculate that the preservation of TLR4 following shock/resuscitation and subsequent treatment with LPS might contribute to the enhanced responsiveness of cells to LPS compared with cells isolated from sham animals. This possibility remains to be tested. Shock/resuscitation may have also altered the innate responsiveness of the TLR4 through conformational changes or through altering its disposition on the cell surface. Clustering of surface receptors by the oxidant stress related to ischemia/reperfusion may contribute to cell signaling, as suggested in studies by Rosette and Karin (31), wherein UV exposure of mammalian cells induced clustering of surface receptors and their activation. In this regard, one intriguing possibility is that oxidant stress may enhance physical approximation of TLR4 with CD14 receptors, an event which was recently shown to promote NF- $\kappa$ B translocation (32). The findings that prolonged exposure to LPS in both sham-derived cells and shock-derived cells caused reduced levels of TLR4 mRNA differ from those reported by others, where TLR4 mRNA appeared to return to baseline levels over time (15). While this may be a result of species differences, it is also possible that prolonged exposure to LPS may have induced a loss of TLR4 from the sur-

face and thus rendered the cells unresponsive to long-term exposure to LPS (15).

In summary, this study provides new insights into the regulation of TLR4 mRNA and protein expression in vivo and in vitro. The data suggest that steady state levels of TLR4 mRNA following exposure to LPS are subject to control by both transcriptional and posttranscriptional mechanisms. Furthermore, the balance of these two mechanisms in vivo may be influenced by antecedent events such as the local microenvironment of the cell. In the context of the trauma patient, the findings suggest a potential target for immunomodulation, even as early as the initiation of resuscitation.

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